PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF SALT TOLERANCE IN BARLEY (*Hordeum vulgare* L.)

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List of Acronyms

agaA	*Aspergillus nidulans* arginase gene
ANOVA	analysis of variance
ATPase	Adenosine 5'-triphosphatase
BARE-1	Barley retroelement 1
BLAST	Basic local alignment search tool
BLASTN	BLAST search a non-redundant nucleotide database
BLASTX	Translate a nucleotide sequence in all six frames and search a protein
BT	Boron toxicity
cDNA	Complementary deoxyribonucleic acids
CGR	comparative growth of root
CGS	comparative growth of shoot
CK	Control
CLT	Claret
CLN	Clansman
dATP	2' Deoxyadenosine 5'-triphosphate
dCTP	2' Deoxycytosine 5'-triphosphate
dbEST	database of EST
DEPC	diethyl pyrocarbonate
dGTP	2' Deoxyguanosine 5'-triphosphate
DNase	Deoxyribonuclease
dNTP	2' Deoxyribonucleotide triphosphate
bp	base pair

dTTP	2' Deoxythymidine 5'-triphosphate
EDAX	energy-dispersive X-ray analysis system
EDXMA	energy-dispersive X-ray microanalysis system
EST	Expressed sequence tags
E-value	Expected frequency
GP	Golden Promise
GPert	Golden Promise erectoides mutation
ICP-AES	inductively coupled plasma-atomic emission epectroscopy
l	liter
IRGA	Infra-red gas analyzer
kb	kilobase
LSD	Least significance difference
LTR	Long terminal repeats
LTSS	Long-term salt stress
min	minute
ml	milliliter
mM	millimole
MMLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
MS	Mannitol stress
nr	Non-redundant database
OLB	old leaf blade
pAGA	Plasmid DNA containing *agaA* sequence

PCR	Polymerase chain reaction
PS I	Photosynthetic system I
PS II	Photosynthetic system II
PT1	5'-$T_{(11)}$A-3'
PT2	5'-$T_{(11)}$G-3'
PT3	5'-$T_{(11)}$C-3'
PT4	5'-$T_{(12)}$CG-3'
PT5	5'-$T_{(12)}$GA-3'
PT6	5'-$T_{(12)}$AG-3'
R1	5'-GTT GCG ATC C-3'
R2	5'-AGC CAG CGA A-3'
R3	5'-TAC AAC GAG G-3'
R4	5'-CTT TCT ACC C-3'
R5	5'-TGG ATT GGT C-3'
R6	5'-GGA ACC AAT C-3'
Rec	Recovery
RNA	Ribonucleic acids
RNase	Ribonuclease
rpm	Rings per minute
RT	Reverse transcription
RT-DD	Reverse transcription and differential display
se	standard error
SEM	scanning electron microscope

SP6	5’-ATTTAGGTGACACTATAGAA
STSS	Short-term salt stress
T7	5’-TAATACGACTCACTATAGGG
TEMED	N’-tetramethylenediamine
µg	microgram
µl	microliter
URT	Untranslated region
V-ATPase	Vacuolar ATPase
YLB	young leaf blade

Dedication

I dedicate this thesis to my wife Yong and my daughter Tina.

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Declaration

I declare that this thesis has been composed by myself, and that the work is my own unless stated otherwise.

Wenxue Wei

Abstract

Two isogenic barley cultivars Golden Promise and Maythorpe were used in this study to investigate physiological and molecular mechanisms of salt tolerance. Using the mRNA differential display technique, 21 cDNA fragments were cloned from young leaves, 10 of them had homologues from GenBank. A leucine zipper transcription factor gene (B15/B20) was up-regulated in Golden Promise and down-regulated in Maythorpe by both short-term and long-term salt stress. The difference in the expression of this gene in response to salinity may regulate the expression of other genes identified from this study in response to salt stress, *e.g.* the Rubisco small subunit, cytochrome B6, chloroplast ATP synthase and V-ATPase B subunit. The V-ATPase B subunit was up-regulated in Golden Promise and down-regulated in Maythorpe under both short-term and long-term salt stress, which indicates the greater potential of Golden Promise in excluding excess Na^+ from the cytoplasm and storage in the vacuoles when compared with Maythorpe. Other genes identified and associated with photosynthesis, *e.g.* genes encoding the Rubisco small subunit, cytochrome B6 and ATP synthase were down-regulated in Maythorpe by both short and long-term salt stress. However, in Golden Promise, the Rubisco small subunit was up-regulated by short-term salt stress and cytochrome B6 was up-regulated by both short and long-term salt stress. The differential expression of the above genes between Golden Promise and Maythorpe in response to salinity was closely related to measured differences

in physiological characteristics. For instance, Golden Promise maintained significantly lower Na^+ concentrations and higher K^+/Na^+ and Ca^{2+}/ Na^+ ratios than Maythorpe in the young leaves with increasing external NaCl concentrations. Golden Promise also showed higher comparative growth of shoot and significantly higher photosynthetic rates than Maythorpe under salt stress.

Chapter 1

General Introduction

1.1 Salinity in agriculture

Soil salinity is a major agricultural problem causing serious reductions in crop growth through the deterioration of soil structure and fertility. About 20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity (Zhu, 2001). About 75 countries have salt related problems in crop production, including Australia, China, Egypt, India, Iraq, Mexico, Pakistan, Russia, Syria, Turkey and the United States (Qadir *et al.*, 2000). These problems have caused huge losses in agriculture, for example in Pakistan alone, about 62% of losses in agricultural incomes are directly associated with soil salinity (Davidson and Ijaz, 1997).

1.2 Causes of soil salinity

A soil having electrical conductivity of the saturated paste extract (EC_e) ≥ 4 deciSiemens per metre (dS m^{-1}) and sodium adsorption ratio (SAR) < 13 is called a saline soil. A soil having EC_e < 4 dS m^{-1} and SAR ≥ 13 is designated as a sodic soil. If a soil has EC_e ≥ 4 dS m^{-1} and SAR > 13, it is categorized as a saline-sodic soil (Soil Science Society of America, 1997). Several factors can cause soil salinization, such as irrigation with poor quality water, high evaporation and intensive application of chemical fertilisers. Global warming will accelerate soil salinization. In saline soils, the major elements include Na^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-}. Na^+ is the most harmful ion among these and most crop plants are sensitive to this ion. If Na^+ accumulates in a

soil, it will result in deterioration of soil structure and stability by replacing other cations such as K^+ and Ca^{2+} from soil particles.

It is predicted that the world population will reach 9.4 billion by 2050 with most of this increase occurring in developing countries. As a consequence of this the global demand for food will double over the period 1990-2030 (Lal, 2000). With this pressure of an increasing population it is necessary to increase agricultural production through both irrigated agriculture and the development of marginal land, which often involves naturally saline land. Irrigation is an important agricultural procedure to increase food supply to feed the increase of population in the last decades. In 1995, about 75% of the world's total irrigated land area (about 190 million hectares) was in developing countries (Lal, 2000), an increase of 60% since the early 1960s (Pinstrup-Anderson *et al.*, 1997). The irrigated land produces a large portion of food, especially in developing countries. Throughout the world, about 17% of irrigated cropland produces 40% of the total food (Postel, 1999).

Unfortunately, irrigation with poor quality water is a major source of soil salinization. In the near future it is likely that limited water resources will not be able to match the rapid increasing requirement for crop production, especially in arid and semi-arid areas. Farmers will have little choice but to use poor quality water to irrigate their crops. The diminishing supply of water together with the use of poor quality water for irrigation results in accumulation of Na^+ ions, this is termed secondary salinization. Unlike marginal land, which has low productivity because of salinity, secondary salinization often occurs on fertile cultivated soils, which directly causes a reduction in agricultural productivity (Eckholm, 1975). Furthermore, the use of saline water for

irrigation is a subject of increasing interest because of the competition between human, industrial and agricultural uses. Moreover there is increased pressure for the disposal of drainage water through reuse. As a result, soil salinization would develop quickly. Tunisia in the Mediterranean area is an example where fresh water resources for agricultural use are rather limited, and extension of irrigated agriculture is mainly possible by using saline water. Thus soil salinity is a serious consequence in that area (Katerji *et al.*, 2000). So using saline soils, including both naturally saline land and the secondary saline soils, to produce food has great potential to meet the high food requirement. Recovery of saline soils by irrigation and drainage is difficult, and therefore a potential target is the breeding of salt tolerant cultivars. Na^+ is one of the most abundant soil cations. Even at a typically low concentration of less than 1 mM (Table 1.1), Na^+ could accumulate to high levels inside plants. This ion remains in a soluble status in the soil solution whilst the other cations such as Ca^{2+} and Mg^{2+} precipitate as calcium and magnesium carbonates when water is evaporated and transpired. Large volumes of transpirational flow of water bring Na^+ from the soil solution into plants (Zhu, 2000).

Table 1.1 Ion concentrations (mM) of seawater and good quality irrigation water (Bressan, 1998).

Ions	Seawater	Good quality Irrigation water
Na^+	457	<2.0
K^+	9.7	1.0
Ca^{2+}	10	0.5-2.5
Mg^{2+}	56	0.25-1.0
Cl^-	536	<2.0
SO_4^{2-}	28	0.25-2.5

1.3 Variation of salt tolerance in different plants

Plants are divided into halophytes and glycophytes depending on their reaction to salinity (Munns *et al.*, 1983). Halophytes are those plants growing under naturally saline conditions (in excess of 200 mmol NaCl), be it seawater, a salt marsh, or a salt-desert; nearly all are angiosperms (Flowers *et al.*, 1986). Understanding of the adaptation of these plants to high salt could be important in providing valuable information to improve the salt tolerance of glycophytes. Halophytes can survive and complete their life-cycles under high salinity because the plants have developed salt tolerance mechanisms due to their evolution under saline conditions. The mechanisms used by halophytes include salt regulation, which restricts the absorption of salt from the soil into the plant. For this there is an early development of Casparian strips within the endodermis under saline conditions (Hajibagheri *et al.*, 1985). A second endodermal layer is developed within the cortex in *Puccinellia peisonis* (Stelzer and Laüchli, 1977), which may act as a barrier to minimize apoplastic transfer of ions to

the xylem. The growth of dicotyledonous halophytes is stimulated by NaCl (Downton, 1982; Burchett *et al.*, 1984; Clough, 1984) and it is presumed that Na^+ substitutes for some of the roles of K^+ within the cell (Flowers and Laüchli, 1983). In addition salt glands, which excrete salt to the leaf surface are found in some species. In salt accumulators high amounts of Na^+ and Cl^- ions are found in the vacuole to maintain cell turgor so as to reduce the exposure of cytosolic enzymes to those ions (Shakespeare, 1999).

Glycophytes or non-halophytes are plants growing in non-saline habitats. They are comparatively limited in their ability to adapt to salinity, because the conditions prevailing during their evolution did not favour the development of such properties (Strogonov, 1964). The growth of these plants in a saline environment always results in reductions in dry matter and yield. A principal difference between halophytes and glycophytes is that halophytes have a greater capacity to survive salt shock. This capacity allows halophytes to more readily establish metabolic steady state for growth by partitioning Na^+ and Cl^- away from the cytoplasm in a saline environment (Braun *et al.*, 1986; Hassidim *et al.*, 1990; Casas *et al.*, 1991; Niu *et al.*, 1993). In contrast, glycophytes restrict ion movement to the shoot by attempting to control ion influx into the root xylem. Halophytes tend more readily to transfer Na^+ from root to shoot and maintain much lower NaCl concentrations in the root than the rest of the plant (Adams *et al.*, 1992).

None the less in glycophytes there is large variation in salt tolerance at low levels of salt between and within different crop species (Table 1.2). Some species can only tolerate very small amounts of salt and even 50 mM NaCl may cause serious damage.

These are called salt sensitive species and examples of such are radish, celery and green bean. Some other non-halophytes such as sugar beet, cotton, spinach and barley can tolerate higher levels of salt, these plants are called salt tolerant non-halophytes. Salt tolerance can also vary between cultivars of the same species. For example, eggplant (*Solanum melongena* L.) is normally salt sensitive. However, cultivars originating from salt affected areas were found to outperform commercial cultivars when grown in a saline environment (Zurayk *et al.*, 1993). An examination of salt tolerance between closely related cultivars (Schachtman *et al.*, 1989; Rogers *et al.*, 1997) may offer an insight into important mechanisms of salt tolerance.

Table 1.2 Salt tolerance of some vegetable and crop plants (Adapted from: Practical Handbook of Agricultural Science, Hanson, 1990).

High salt tolerance	Medium salt tolerance	Low salt tolerance
Table beet	Tomato	Radish
Kale	Broccoli	Celery
Asparagus	Cabbage	Green bean
Spinach	Bell pepper	Field bean
Barley	Cauliflower	White clover
Sugar beet	Lettuce	Meadow foxtail
Rape	Sweet corn	Alsike clover
Cotton	Potato	Red clover
Bermudagrass	Carrot	Ladino clover
Rhodesgrass	Onion	
Western wheatgrass	Pea	
Birdsfoot trefoil	Squash	
	Cucumber	
	Rye	
	Wheat	
	Oat	
	Rice	
	Sorghum	
	Corn	
	Flax	
	Sunflour	
	Castor bean	
	Sweet clover	
	Perennial ryegrass	
	Strawberry clover	
	Dallis grass	
	Sudan grass	
	Alfalfa	
	Tall fescue	
	Orchardgrass	
	Meadow fescue	
	Reed canarygrass	
	Big trefoil	
	Smooth bromegrass	

1.4 Physiological effects of salinity

The effect of soil salinity on plant growth can be divided into two phases. One is short term and represents the period from the beginning of salt shock to a few hours or weeks. At this stage salt stress is dominated by osmotic disturbance, which causes the loss of turgor in plant cells, stomatal closure and reduction of photosynthesis. As a consequence, plant growth is inhibited. The other is the long term phase covering a period of some weeks after phase one. At this stage salinity stress is dominated by ion toxicity and ionic imbalance, plants may express symptoms like potassium deficiency and leaf necrosis (Munns, 1993; Munns *et al.*, 1995; Neumann, 1997).

Salt tolerance can be assessed at different stages throughout the life cycle of plants. Germination is a common event used to exhibit differences of salt resistance between plant species. Since germination is the first critical stage of the plant life-cycle, the plant is very sensitive to the external nutrient supply. Therefore, germination is often used to study the early reactions of salt tolerance. The advantage of utilising germination as an indicator is that it can be used to screen a large number of plant varieties for salt tolerance in a short period and to investigate the early responses of plants to high salinity. The disadvantage is that, in some circumstances, this stage is too early to judge whether a plant is salt tolerant because the response may change later (Noble, 1983). For example, salinity tolerance of sugar beet was found to be lowest during germination compared to other growth stages (Bernstein and Hayward, 1958).

Plant dry matter weight is an alternative to estimate the effect of salt on vegetative growth. Plants may be assessed as being salt-tolerant or salt-sensitive from their dry

matter production after extended periods of growth under saline conditions. The disadvantage of studying this stage is similar to that of germination, where dry weight may not adequately cover all of the salt tolerance characters.

Yield is the most important trait used to measure performance in a saline environment, because this trait can reflect the characteristics of the whole life cycle including both phases. Richards *et al.* (1987) described an inverse linear relationship between salt concentration in the soil and yields of dry matter and grain in different cultivars of hexaploid and tetraploid wheat, triticale and barley. The disadvantage of measuring yield is that it is laborious, costly and time consuming.

Leaf symptoms caused by high salinity could be an indicator for salt tolerance, because salt normally results in leaf desiccation, foliar damage, necrosis and death. These visual symptoms can easily be used to estimate the differential response between and among plant species under salt stress. Potential problems are that the symptoms are usually a mixture including Na^+ and Cl^- toxicity, K^+ deficiency and drought. Investigation of leaf symptoms may not be quantitative, therefore it is difficult to compare the results from different plant species and researchers.

Soil salinity affects photosynthesis in plants. The rate of photosynthesis of many plant species is reduced in the presence of NaCl, which is linked to salt induced reduction of plant growth. The reduction of the rate of photosynthesis has been attributed to salt damage of the photosynthesising tissues (Yeo *et al.*, 1985) because correlations can often be shown between tissue ion concentrations and net carbon dioxide (CO_2) exchange rate (Downton, 1977; Yeo *et al.*, 1985; Rawson, 1986). Stomatal

conductance is also seriously decreased under salt stress (Heuer and Plaut, 1981; Robinson *et al.*, 1983).

Tissue Na^+ concentration is another important criterion in the assessment of salt tolerance. Growing in a saline environment, a plant can accumulate Na^+ in both shoot and root. The variations of Na^+ concentrations between varieties can be used to distinguish salt tolerant and salt sensitive cultivars (Greenway and Munns, 1980; Gorham *et al.*, 1985; Neumann, 1997; Pakniyat *et al.*, 1997a), as in glycophytes, the ability to limit shoot Na^+ concentration is an important mechanism of salt tolerance.

Ionic imbalance is an important characteristic at the second phase of salt stress. This is because Na^+ uptake results in the reduction in the absorption of other nutrients such as Ca^{2+} and K^+. K^+/Na^+ ratios are treated as an indicator in salt tolerance studies of plants. Salt tolerant varieties have higher K^+/Na^+ ratios than salt sensitive varieties (Gorham, 1990; Gorham *et al.*, 1990a; Schachtman *et al.*, 1991). Therefore K^+/Na^+ ratio is employed as another useful character to understand salt tolerance mechanisms.

1.5 Plant adaptation to soil salinity

1.5.1 Physiological adaptation to salt stress

1.5.1.1 Ion homeostasis in NaCl stress environments

Homeostasis can be defined as the tendency of a cell or an organism to maintain an internal steady state. When plant cells are exposed to salinity, their kinetic steady states of ion transport for Na^+, Cl^- and other ions, such as K^+ and Ca^{2+}, are disturbed (Binzel *et al.*, 1988). High apoplastic levels of Na^+ and Cl^- alter the aqueous and ionic thermodynamic balance, resulting in hyperosmotic stress, cellular ionic imbalance and

toxicity. Thus, it is vital for the plant to re-establish cellular ion homeostasis for metabolic functioning and growth to adapt to the saline environment.

1.5.1.2 Na^+ and Cl^- uptake

Little is known about intracellular uptake and vacuolar compartmentation of Cl^- (Binzel *et al.*, 1988). Generally, the extreme inside negative membrane potential across the plasma membrane is a substantial thermodynamic barrier to Cl^- influx. Chloride uptake could be considered in two cellular contexts. Immediately after the salt shock, the H^+ electrochemical gradient is altered. Influx of Na^+ dissipates the membrane potential, then the external Cl^- ions could flow down the chemical gradient across plasma membranes through an anion channel (Hedrich, 1994; Skerrett and Tyerman, 1994; Czempinski *et al.*, 1999). However, after steady-state conditions are re-established, including establishment of an inside negative plasma membrane potential, Cl^- influx would be an active process mediated by a Cl^--H^+ symporter even at relatively high external concentrations (Poole, 1988). Cl^- partitioning into vacuoles from the cytoplasm is an essential adaptation for NaCl tolerance. Vacuolar compartmentation of Cl^- may be through a channel or a carrier driven by a H^+ pump, for example, Cl^- may transport as a counter ion of H^+. An alternative possibility is a H^+/anion antiporter that would actively transport Cl^- across the tonoplast (Rea and Sanders, 1987; Sze *et al.*, 1999).

When the NaCl concentration increases in the surrounding environment, the high extracellular level of Na^+ (relative to the cytosol) and negative membrane potential establishes a steep thermodynamic gradient for Na^+ influx. However, the precise transport system responsible for Na^+ uptake into the cell is still unknown.

Physiological data indicates that Na^+ competes with K^+ for intracellular influx because these cations are transported by common proteins (Niu *et al.*, 1995; Amtmann and Sanders, 1999). Plant roots utilize two systems for K^+ acquisition. System 1 has a high affinity for K^+ to allow uptake at low K^+ concentrations and it is not inhibited by Na^+ (Rains and Epstein, 1967). System 2 has low affinity for K^+ and mediates uptake at higher external K^+ concentrations (mmol) and has a less pronounced K^+/Na^+ selectivity. Na^+ influx into plants is likely to occur *via* the low-affinity rather than the high-affinity K^+ uptake system. Schachtman *et al.* (1991) suggested that Na^+ uptake may occur through non-specific cation channels, which transport common cations such as K^+, Na^+, Rb^+, Ca^{2+}, Cs^{2+} *etc.* Cation channels show poor overall selectivity and are permeable to Na^+ (Amtmann *et al.*, 1997; Roberts and Tester, 1997; Tyerman *et al.*, 1997). These channels are therefore potential candidates for Na^+ entry in saline conditions. If a non-selective cation channel is a pathway of Na^+ entry in saline conditions then its down-regulation would enhance salinity tolerance. Maathuis and Sanders (2001) also suggested that in *Arabidopsis* root Na^+ uptake could be regulated by cyclic nucleotides.

1.5.1.3 Maintenance of low cytosolic Na^+ concentrations

Na^+ is a non-essential element to most plants with the exception of some halophytes and C_4 species. Cytosolic Na^+ concentration in excess of about 100mM would be cytotoxic (Amtmann and Sanders, 1999), therefore most Na^+ ions have to be removed from the cytosol. In plants, the main mechanism for Na^+ extrusion is mediated by the plasma membrane H^+-ATPase (Sussman, 1994). The H^+-ATPase uses the energy of ATP hydrolysis to pump H^+ out of the cell, generating H^+-electrochemical potential gradients across the plasma membrane. This proton motive force is important to

manipulate Na^+ and Cl^- extrusion and Cl^- influx. The gradients established could facilitate between 10^2- to 10^3- fold lower concentrations of Na^+ and Cl^- in the cytosol relative to the apoplast or the vacuole (Hasegawa *et al.*, 2000). Even at seawater concentrations of Na^+ and Cl^-, energy-dependent transport of these ions into the apoplast and vacuole (so that toxic levels do not accumulate in the cytosol) can be facilitated by the capacity of the H^+ electrochemical potential gradients established across the plasma membrane and tonoplast. Wu and Seliskar (1998) indicated that there was a significant increase of plasma membrane H^+-ATPase (PM-ATPase) activity when the callus of the salt marsh plant *Spartina patens* was grown on media containing NaCl. With salt-tolerant and salt-sensitive lines of spring wheat (*Triticum aestivum* L.), the PM-ATPase activity in the shoots of tolerant plants was stimulated by salt (Ayala *et al.*, 1997).

Na^+ efflux across the plasma membrane and compartmentalisation into vacuoles or pre-vacuoles is a major pathway to keep low Na^+ concentrations in the cytoplasm, this procedure is mediated presumably by Na^+/H^+ antiporters (DuPont, 1992). Na^+/H^+ antiporters are transport proteins that exchange Na^+ for protons across a membrane and partition Na^+ into vacuoles (Krulwich, 1983; Grinstein *et al.*, 1989). Both a proton gradient (ΔpH) and a Na^+ gradient ($\Delta\mu Na^+$) can serve as the driving force. In plants, Na^+/H^+ antiporters were physiologically detected in isolated tonoplasts from *Beta vulgaris* L. (Blumwald and Poole, 1985), *Plantago* roots (Staal *et al.*, 1991), *Hordeum vulgare* roots (Garbarino and DuPont, 1988) and in intact vacuoles from *Catharanthus* cells (Guern *et al.*, 1989). Under salt stress, two forms of up-regulation of the Na^+/H^+ antiporter were detected in barley and sugar beet. In barley the antiport activity was not detected unless the plants were exposed to NaCl (Garbarino and DuPont, 1988;

Staal *et al.*, 1991). In sugar beet, the antiport activity was increased by NaCl (Blumwald and Poole, 1987). The activity of the Na^+/H^+ antiporter may be an important mechanism contributing to salt tolerance in plants, but further molecular and genetic evidence is still required to support physiological data.

1.5.1.4 Ca^{2+} and K^+ homeostasis

Ca^{2+} is an abundant element in soils and usually present in sufficient amounts to meet plant needs. This element constitutes 0.1-2.0% of the dry weight of plants and structurally stabilizes membranes and cell walls (Marschner, 1995). Ca^{2+} also has signalling functions in plants. Under high salinity, Ca^{2+} can have a protective effect on plant growth and survival (Greenway and Munns, 1980; Läuchli, 1990; Rengel, 1992). The role of Ca^{2+} in NaCl stress adaptation is complex and not well defined. Externally supplied Ca^{2+} reduces the toxic effects of NaCl through an unknown function that preserves K^+/Na^+ selectivity (Zhong and Läuchli, 1994). Under high salinity, Na^+ was shown to displace Ca^{2+} from the plasma membrane of cotton root hairs resulting in a change in plasma membrane permeability that was detected as a leakage of K^+ (Cramer *et al.*, 1985). Moreover, Ca^{2+} inhibits inward-rectifying K^+ channels, that may reduce the Na^+ influx mediated by the low-affinity component of K^+ uptake (Schroeder *et al.*, 1994). Cytosolic Ca^{2+} levels are usually maintained at 100 to 200 nM by active transport, and small increases in concentration typically initiate very specific signal transduction cascades (Bush, 1995). NaCl causes a rapid increase in cytosolic Ca^{2+} that probably acts as a general stress signal (Lynch *et al.*, 1989), although it is not clear if this increase is an effector of salt tolerance. The resultant transient Ca^{2+} increase potentiates stress signal transduction and leads to salt adaptation (Knight *et al.*, 1997; Sanders *et al.*, 1999).

K^+ plays central roles in plant growth and development, including maintenance of turgor pressure, leaf and stomatal movement, and cell elongation (Smart *et al.*, 1996; Maathuis *et al.*, 1997; Thiel and Wolf, 1997). High salinity can cause K^+ deficiency in plants. Salt tolerant plants are generally associated with selective uptake of K^+ into the shoot (Davis, 1984; Schachtman *et al.*, 1989; Gorham *et al.*, 1990) and are able to maintain higher K^+/Na^+ ratios, which is an important feature in salt tolerance (Flowers *et al.*, 1986; Gorham, 1990; Ayala *et al.*, 1997).

1.5.1.5 Water channels

High salinity caused a considerable reduction in water permeability in the cortex of maize roots (Tyerman *et al.*, 1989; Azaizeh *et al.*, 1992), reducing the osmotic water permeability by as much as fivefold. Changes in the osmotic water permeability were reflected in changes in root hydraulic conductivity due to the fact that most of the water was flowing around cells (Azaizeh and Steudle, 1991). Conceivably, such changes may be caused by reducing the opening of water channels or by a change in their number.

A group of membrane channel forming proteins aptly named "aquaporins" facilitate the passive movement of water across membranes. Aquaporins belong to the Major Intrinsic Protein (MIP) family of channels conserved in animals, plants and micro-organisms, that also includes glycerol and ion transporters (Maurel, 1997; Schäffner, 1998). In plants, aquaporins fall into two distinct subclasses of the MIP family, the putative plasma membrane intrinsic proteins (PIPs) and the putative tonoplast intrinsic proteins (TIPs) (Yamada *et al.*, 1995).

Water channels are assumed to largely contribute to cellular hydraulic conductivity (L_p), and thus play a significant role in the control of transmembrane water movement during growth, development and stress responses of plants. In *Mesembryanthemum crystallinum*, several MIP homologs (MipA through Mip E) have been identified by differential screening of a root cDNA library constructed from salt-stressed plants (Yamada *et al.*, 1995). All of these genes were shown to encode proteins that fall into the PIP subfamily of putative aquaporins. Homologues of a tonoplast transmembrane channel protein have been identified in *Arabidopsis thaliana* and pea (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Messenger RNA for the pea homologue was rapidly accumulated following the onset of desiccation. Induction of water-specific transporters during the initial stages of increasing salinity in the environment may complement those mechanisms leading to increased vacuolar osmolyte concentration and osmoregulation (Bartels and Nelson, 1994).

1.5.2 Biochemical adaptation to salt stress

1.5.2.1 Osmoregulatory substances

Osmoregulation plays an important role in salt tolerance in plants. Osmotic stress causes the accumulation of metabolites that act as "compatible" solutes, *i.e.* they do not inhibit normal metabolic reactions. With accumulation proportional to the change of external osmolarity within species-specific limits, protection of structures and osmotic balance supporting continued water influx (or reduced efflux) are accepted functions of osmolytes. Frequently observed metabolites with an osmolyte function are sugars (mainly sucrose and fructose), sugar alcohols (glycerol, methylated inositols) and complex sugars (trehalose, raffinose, fructans). In addition, ions (K^+) or charged metabolites, *e.g.* glycine betaine, dimethyl sulphonium propionate (DMSP),

proline and ectoine (1, 4, 5, 6 - tetrahydro - 2 - methyl - 4 - carboxyl pyrimidine), are encountered. The accumulation of these osmolytes is believed to facilitate "osmotic adjustment", by which the internal osmotic potential is lowered and may then contribute to tolerance (Delauney and Verma, 1993; Louis and Galinski, 1997).

Compatible solutes at high concentrations can reduce inhibitory effects of ions on enzyme activity (Solomon *et al.*, 1994). This will cause an increase in the thermal stability of enzymes (Galinski, 1993), and prevent the dissociation of enzyme complexes, for example, the oxygen-evolving complex of photosystem II (Papageorgiu and Murata, 1995).

Glycine betaine (GB) can accumulate in some plant species in response to environmental stresses. In higher plants, it is synthesised from choline *via* betaine aldehyde using choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BALDH) in chloroplasts (Rathinasabapathi *et al.*, 1997). In *Arabidopsis* in which *codA* (which encodes choline oxidase from *Arthrobacter globiformis*) has been expressed, the transgenic plants were more tolerant to NaCl (Hayashi *et al.*, 1997). Expression of *Atriplex hortensis* BALDH in rice was shown to increase GB content and salinity tolerance (Guo *et al.*, 1997). Genetic evidence that GB improves salinity tolerance has been obtained for barley and maize (Grumet and Hanson, 1986; Rhodes *et al.*, 1989).

Trehalose is an effective desiccation protectant in animals and microbes but is rare in vascular plants with the exception of a few desiccation tolerant species. Elevated trehalose in plants is toxic, however, short term exposure to exogenous trehalose

appears to decrease salt damage to rice seedlings (Garcia *et al.*, 1997; Goddijn *et al.*, 1997; Roero *et al.*, 1997).

Mannitol is accumulated by a wide range of species in response to drought and salinity (Stoop *et al.*, 1996). Mannitol synthesising ability was conferred in transgenic tobacco by introducing the *E. coli mtlD* gene encoding mannitol dehydrogenase. These plants accumulated modest amounts of mannitol but were said to be more salt tolerant. In the absence of salt, control and transformed plants have similar height and fresh weight gains (Tarczynski *et al.*, 1993). In the presence of 250 mM salt, the mannitol-containing plants have a growth advantage over control plants in terms of better height gain, less fresh weight loss and more new leaf and root production. It also has been suggested that compatible solutes, including mannitol, could be antioxidants by scavenging hydroxyl radicals (·OH) (Smirnoff and Cumbes, 1989; Orthen *et al.*, 1994). Chloroplast-targetted mannitol accumulating tobacco has been used to test this hypothesis. Mannitol accumulation did not affect photosynthesis. The transgenic plants were more tolerant to ·OH generated in chloroplasts by methyl viologen treatment. The ·OH content of transgenic plants was also lower (Shen *et al.*, 1997) suggesting that the protection could result from ·OH scavenging by mannitol.

Proline is often synthesized by plants in response to osmotic stress. For example, tobacco cells adapted to NaCl accumulate proline to 80-fold higher levels, and this is largely accounted for by increased synthesis (Rhodes and Handa, 1989).

1.5.2.2 Antioxidants and stress resistance

Salt stress can generate active oxygen species, including superoxide (O_2^-), hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Lee *et al.*, 2001). These active oxygen species are thought to play an important role in inhibiting plant growth. As a response, the plants need to scavenge harmful radicals to maintain normal growth. Normally, a highly efficient antioxidant defence system is present in plant cells, and the antioxidants can be divided into two classes: (1) nonenzymic constituents, including lipid-soluble and membrane-associated tocophenols, and (2) enzymic constituents, including superoxide dismutase (SOD), catalase, peroxidase, ascorbate peroxidase (APX) and glutathione reductase (GR) (Foyer, 1993). The primary scavenger in the detoxification of active oxygen species in plants is SOD, which is a group of metalloenzymes that converts superoxide to H_2O_2 and O_2 and protects cells against superoxide-induced oxidative stress (Asada and Kiso, 1973; Fridovich, 1986). In rice (*Oryza sativa* L.) salt stress enhanced the activities of the SOD, APX and peroxidase systems (Lee *et al.*, 2001). Dionisio-Sese and Tobita (1998) demonstrated that the salt-tolerant rice variety Pokkali had higher SOD and peroxidase activities than the salt sensitive varieties, Hitomedore and IR28. They suggested that salt tolerant varieties exhibit protection mechanism against increased radical production by maintaining the specific activity of antioxidant enzymes. In pea (*Pisum sativum* L.) and tomato (*Lycopersicon pennellii*) similar results have been also found (Hernández *et al.*, 2000; Mittova *et al.*, 2000).

1.5.3 Molecular Mechanisms of Salt Tolerance

1.5.3.1 Salt Stress Tolerance genes

In the last two decades, using powerful molecular techniques a number of salt tolerance genes and gene products have been identified from plants, animals and fungi, some of which have been functionally characterised. Generally, salt stress tolerance genes can be divided into functional genes that lead to adaptation to salinity and regulatory genes that control the degree and timing of expression of the functional genes (Hasegawa *et al.*, 2000).

Regulatory genes or transcription factors have always been predicted to play a major role in the control of plant responses to salt stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu *et al.*, 1997). These factors participate in the activation of stress-inducible genes and presumably lead to osmotic adaptation (Sheen, 1996; Abe *et al.*, 1997; Liu *et al.*, 1998; Winicov and Bastola, 1999). Since the promoters that are controlled by these transcription factors are responsive to several environmental signals, it is not clear which transcription factors, if any, function only in salt stress responses, or if salt-specific transcriptional regulation alone is a required component of salt tolerance in plants. Over-expression of the zinc finger transcription factor ALFIN1 activates *MsPRP2* (NaCl responsive gene) expression and increases salt tolerance of alfalfa (Winicov and Bastola, 1999). Two *Arabidopsis* transcription factor-encoding cDNAs *STO* (salt tolerance) and *STZ* (salt tolerance zinc finger) suppressed the Na^+/Li^+ sensitive phenotype of a calcineurin-deficient mutant (Lippuner *et al.*, 1996). Calcineurin is a calcium-dependent protein phosphatase that mediates the high-salt-induced expression of the ENA1/PMR2 gene (Na^+-ATPase gene of *Saccharomyces cerevisiae*) in yeast. *STZ*- mediated salt tolerance is at least

partially dependent on *ENA1*, implying a function that involves regulation of the Na^+-ATPase, whereas *STO* function is independent of *ENA1*. *AtGSK1* was isolated from *Arabidopsis* as a suppressor of the NaCl-sensitive phenotype of a calcineurin-deficient mutant (Piao *et al.*, 1999). *AtGSK1* expression induced transcription of yeast ENA1, which encodes a P-type ATPase responsible for Na^+/Li^+ efflux across the plasma membrane.

Some structural genes and their gene products that respond to salt stress have been identified from both fungi and plants. Those genes and their products mediate many of the mechanisms of salt tolerance, *i.e.* ion homeostasis, osmolyte biosynthesis, scavenging of reactive oxygen species (ROS), water transport (Hasegawa *et al.*, 2000). Recently, plant cDNAs encoding NHE-like proteins (Na^+/H^+ exchanger) were isolated that can functionally complement a yeast mutant deficient for the endomembrane Na^+/H^+ transporter, NHX1 (Apse *et al.*, 1999; Fukuda *et al.*, 1999; Gaxiola *et al.*, 1999). Over-expression of an NHE-like antiporter substantially enhanced the salt tolerance of *A. thaliana*, confirming the function of the transporter in Na^+ compartmentation (Apse *et al.*, 1999) and transgenic tomato by over-expressing a vacuolar Na^+/H^+ antiport were able to grow in the presence of 200 mM NaCl (Zhang and Blumwald, 2001).

A collection of inward-rectifying potassium channels (KAT1, AKT1, KST1) have been identified from *A. thaliana* and *Solanum melongena* from cDNA clones complementing yeast mutants that lack endogenous potassium transporters and permit low-affinity (200 μM external potassium) growth (Smart *et al.*, 1996). HKT1 is a cDNA for a membrane protein from wheat roots whose properties correspond with the

classical 'high-affinity' K^+-transport. At toxic activities of Na^+, HKT1 mediates low-affinity Na^+-uptake while K^+-uptake is blocked (Gassmann *et al.*, 1996). However there is limited knowledge of how channels/transporters regulate intracellular K^+ levels (Smart *et al.*, 1996).

In the last two decades, a long but incomplete list of salt stress-responsive genes has been produced by molecular studies (Zhu *et al.*, 1997). Several factors could be related to the limited success of the molecular approach in elucidating salt tolerance mechanisms. Firstly, because most physiological work has been performed on genetically unrelated varieties or species, molecular and genetic analysis could be complicated by different genetic backgrounds. Secondly, like other stresses, salt stress can cause changes in the expression of many genes, some are general stress responses and some are related to high salinity, but the key changes may be unknown. Finally, many genes related to salt stress have been identified using single cell models. These genes and their gene products are quite important in understanding salt tolerance mechanisms in plants (Hasegawa *et al.*, 2000). However the response at the whole-plant level to salt stress could be very different in comparison with the single cell because of the complex relationships between and within cells, tissues and organs. More recently it has been recognised that the processes that are advantageous to a single cell in an aqueous medium may be lethal to a cell in a leaf in its natural environment (Yeo, 1998). Therefore, molecular studies with higher plant models will be important in understanding salt tolerance mechanisms at the whole plant level.

1.5.3.2 Genetics of salt tolerance

Salt tolerance is a complex trait involving responses to cellular, osmotic and ionic stresses and their consequent secondary stresses (*e.g.* oxidative stress) and whole plant co-ordination. Past efforts to improve plant tolerance to high salinity through breeding and genetic engineering have had limited success owing to the genetic complexity of stress responses. Progress is now anticipated through comparative genomics studies of an evolutionarily diverse set of model organisms. Techniques such as high-throughput analysis of expressed sequence tags (EST), large-scale parallel analysis of gene expression, targeted or random mutagenesis and gain-of-function or mutant complementation have been adopted (Cushman and Bohnert, 2000). The discovery of novel genes, determination of their expression patterns in response to abiotic stress and an improved understanding of their roles in stress adaptation will provide the basis of effective engineering strategies leading to greater stress tolerance. This will also better explain the physiological basis of salt tolerance.

Pleiotropic mutations that change ion transport characteristics, *e.g.* to restrict Na^+ and or to enhance K^+ influx, may also result in enhanced salt tolerance in plants. These include soybean mutants that appear to exclude Cl^- (Abel, 1969), fern mutants with altered K^+ transportation (Warne *et al.*, 1996), and several *Arabidopsis* mutants including those with altered Na^+/H^+ antiporter, protein kinase or Ca^{2+} binding functions (Saleki *et al.*, 1993; Zhu *et al.*, 1998; Tsugane *et al.*, 1999). Saleki *et al.* (1993) selected three *RS* (Resistant to Salts) mutants of *Arabidopsis* that are capable of germination under saline conditions. The *RS* mutants show enhanced tolerance to not only NaCl but also KCl, K_2SO_4, LiCl and mannitol, indicating that the mutants are primarily osmotolerant. Although the *RS* mutants germinate under high salinity,

seedlings and mature plants are not more salt tolerant than the wild type. Barley and tobacco mutants with salt tolerance characters overaccumulate proline (Kueh and Bright, 1982; Sumaryati *et al.*, 1992). These observations imply that different salt tolerance mechanisms may operate during seed germination and subsequent plant growth.

The unicellular yeast, *S. cerevisiae*, has been used as an alternative model to study salt stress in plant. From this model two important pathways for salt stress tolerance have been identified. One was the HOG1 pathway for adaptation to hyperosmotic stress and the other was the calcineurin pathway for ionic stress. Both pathways suggested possible salt tolerance mechanisms in higher plants (Zhu, 2001).

A. thaliana has emerged recently as an excellent model system to study plant salt tolerance (Zhu, 2000), which has obvious advantages over yeast because it is a real plant and the mechanisms of salt tolerance in whole-plant integration can be studied. The availability of the entire genome sequence (http://mips.gsf.de/proj/thal/db/index.html) makes the *Arabidopsis* system even more powerful and attractive. Application of the *Arabidopsis* model system has yielded a regulatory pathway for ionic homeostasis under salt stress (Zhu, 2000). The pathway was discovered through the cloning of the salt overly sensitive (*SOS*) genes. Mutations in the *SOS* genes resulted in *Arabidopsis* plants more sensitive to Na^+ stress. However it is still possible that novel processes or mechanisms unique to naturally stress-tolerant plants could be difficult to study with *Arabidopsis*. Hence, a genetic model system(s) will be needed that is based on naturally tolerant plants (Zhu, 2001).

1.6 Barley and salt tolerance

Barley (*H. vulgare* L.) can be used as a model crop species to study salt tolerance. Barley is one of the most salt tolerant crops (Table 1.2) (Ayers *et al.*, 1952; Bole and Wells, 1979). It is a diploid species with a relatively small number of chromosomes (n=7). A wide range of genetic stocks are available including barley chromosome addition lines, near isogenic lines, recombinant inbred lines, doubled haploids, trisomics, translocations, mutants and wild species variants.

Barley is widely grown in many climates of the world. Early spring types can grow within the Arctic Circle, further north than any other cereal. Barley can also be grown in the hot climates of the Sahara in Africa, the high plateaux of Tibet and tropical plains of India. Barley is relatively tolerant of alkaline soils, drought and extremes of temperature. Barley has been grown successfully in fields that irrigation has rendered unsuitable for other crops. Variations in sensitivity to salt stress exists among barley cultivars (Yeo and Flowers, 1989; Forster *et al.*, 1994).

Golden Promise is one of the *GPert* mutants, which were produced by gamma-ray irradiation of the barley, *H. vulgare* L. cv. 'Maythorpe' (Fig. 1.1) in the late 1950s by the English plant breeding company Miln Marsters Ltd. The aim was to induce a shorter straw length, good malting-quality barley, which would be better suited to combine harvesting. Golden Promise was released commercially in the UK in 1966 (Pakniyat *et al.*, 1997a).

Fig. 1.1 Pedigree of Golden Promise (Forster *et al.*, 1994).

Goldthorpe×Maja
↓
Maythorpe
↓
γ-ray irradiation
(6-24 rad)
↓
line 759/4
↓
selfing generations
↓
Golden Promise

Golden Promise offered barley growers a unique combination of highly desirable agronomic characters such as earliness of ripening, short stiff straw, easy combining, good resistance to grain and ear loss as well as to grain splitting and good malting potential. It soon became evident that Golden Promise was agronomically more suited to the environmental conditions of Northern Britain than previous cultivars. In 1968 Golden Promise was introduced to the recommended list of cereals for Scotland, where it dominated the Scottish barley acreage from the 1970s to the mid 1980s. Pakniyat *et al.* (1997a) showed that the *GPert* mutation has a positive effect on salt tolerance in reducing shoot Na^+ concentration compared to non-*GPert* lines (Pakniyat *et al.*, 1997b) and Golden Promise was found to be salt tolerant compared to its parent Maythorpe (Forster *et al.*, 1994; Pakniyat *et al.*, 1997a). These barley cultivars therefore provide an attractive model system for the investigation of the physiological and molecular basis of salt tolerance in two genetically related varieties of a major crop plant. The disadvantages of the 'correlative' approach of traditional physiological studies where cause and effect are indistinguishable are hence reduced. A relatively

simple genetic change that controls salt response in addition to growth habit can be studied in the otherwise isogenic backgrounds of two cultivars of a major crop plant.

1.7 Aims and objectives

Golden Promise and Maythorpe exhibit differences in salt tolerance largely through the maintenance of a low Na^+ content in the shoots of the former variety (Forster *et al.*, 1994; Pakniyat *et al.*, 1997a,b). However, the mechanisms as to how this is brought about are poorly understood. The main aim of this study was to explore the differences in the uptake and distribution of Na^+ and Cl^- at the organ, tissue and cell level in these two varieties and use this knowledge to further explore physiological, biochemical and molecular responses to high salt concentrations.

1) To investigate differences in the Na^+ and Cl^- uptake and distribution in different tissues of Golden Promise and Maythorpe.

2) To study the ionic imbalance arising from high external Na^+ and Cl^- concentrations and their effects on the distribution of other ions, *e.g.* K^+, and Ca^{2+}. These studies may help to understand how ion imbalance and ion distribution are related to salt tolerance.

3) To isolate the genes that are differentially expressed in Golden Promise and Maythorpe under saline conditions through the mRNA differential display technique (Liang and Pardee, 1992; Liang *et al.*, 1992) and to characterise the expressed gene(s) in relation to salt tolerance.

Chapter 2

Ion homeostasis

2.1 Introduction

Salinity may decrease biomass production due to low water potential, specific ion toxicity, or ion imbalance (Greenway and Munns, 1980). Plants protect themselves from sodium chloride (NaCl) toxicity by minimizing Na^+ uptake and transport to the shoots (Cramer *et al.*, 1989). This is particularly evident in the *Triticeae* (Greenway and Munns, 1980; Davis, 1984; Gorham *et al.*, 1985; Schachtman *et al.*, 1989) and specifically in barley where Na^+ exclusion from the shoots has been shown to be an important mechanism in salt tolerance (Forster *et al.*, 1994; Pakniyat *et al.*, 1997a). Schachtman *et al.* (1989) found in wheat that in addition to Na^+, low Cl^- accumulation in shoots was related to salt tolerance. An excess of Na^+ in the root media may result in a passive accumulation of this ion in root and shoot tissues, and a high Na^+/K^+ ratio will lead to metabolic disorders such as reductions in protein synthesis and enzymatic activities (Brady *et al.*, 1984). High Na^+ disrupts K^+ nutrition in spinach (Chow *et al.*, 1990) and so maintainance of adequate K^+ concentrations in cells is crucial for continued growth under saline conditions. K^+ is needed not only for cell turgor to drive cell expansion, but also as a cofactor for many enzymes (Leigh and Wyn Jones, 1984). Saline conditions were shown to induce potassium deficiency in spinach and maize (Chow *et al.*, 1990; Botella *et al.*, 1997) and K^+/Na^+ selectivity has been found to be an important factor in the tolerance of cotton (Cramer *et al.*, 1986). In addition, high levels of Na^+ can displace Ca^{2+} from root membranes, changing their integrity (Cramer *et al.*, 1985) and thus affecting the selectivity for K^+ uptake (Cramer *et al.*, 1987).

Calcium was shown to reduce the toxic effect of high NaCl in young barley seedlings (Cramer *et al.*, 1989). Elevated Ca^{2+} levels may protect plants from NaCl toxicity by reducing the displacement of membrane-associated Ca^{2+} (Cramer *et al.*, 1985) and Na^+ uptake and transport to the shoots of barley, wheat and strawberry (Cramer *et al.*, 1989; Kinraide, 1999; Kaya *et al.*, 2002), or by a combination of these effects. Calcium plays an important role in salt tolerance by improving K^+ uptake under NaCl salinity (Cramer *et al.*, 1985; 1987), effectively increasing the K^+/Na^+ ratio in tissues. Similarly, improvement in salt tolerance in wheat, barley and tomato was observed when phosphorus concentration was increased in the external medium (Manchanda *et al.*, 1982; Gibson, 1988; Awad *et al.*, 1990). Increasing levels of phosphorus were also shown to improve the tolerance of barley to NaCl (Al-Karaki, 1997). Ionic imbalances induced by high NaCl concentrations are therefore important in understanding the physiological basis of tolerance to salinity.

A study of ion relations in a near-isogenic background by comparing the cultivars Maythorpe and Golden Promise may help to understand the physiological basis of the enhanced salt tolerance of Golden Promise. In addition to Maythorpe and Golden Promise, further two barley cultivars, Claret and Clansman, were used to broaden the comparison. Clansman is another *GPert* mutant, which contained the least Na^+ in the shoot and Claret is a non-*GPert* cultivar, which contained the highest Na^+ in the shoot in a comparative study of *GPert* and non-*GPert* barley cultivars under 175 mM external NaCl by Pakniyat *et al.* (1997a).

The objective of this part of the project was to compare the ion relations between Golden Promise and Maythorpe in addition with Clansman and Claret to investigate

varietal differences in ion concentrations and distributions in different tissues of the above cultivars with increasing salinity, and to understand the relationships of ion homeostasis and salt tolerance.

2.2 Materials and methods

2.2.1 Plant growth experiments

2.2.1.1 Plant experiment one

Four spring barley cultivars Maythorpe, Golden Promise, Clansman and Claret (seed supplied by the Scottish Crop Research Institute) were used in this experiment. Seeds were soaked on wet filter paper in petri dishes for one day at room temperature, after which petri dishes were kept at 4 °C for 2 days prior to maintaining at room temperature for 1-2 days depending on the rate of germination. Germinated grains were transferred into 10 x 1.5 cm diameter plastic tubes (for support) partially filled with vermiculite and having a hole cut in the bottom to allow the roots to grow through. The seed was positioned about 5 cm from the bottom of the tube so that roots were immersed in nutrient solution at all stages of growth. One seed was placed in each tube and four tubes were placed in each black tray containing 2.5 l of distilled water. After 5 days, the water was replaced by half-strength Hoagland and Arnon nutrient solution (Table 2.1). After a further 5 days, full-strength Hoagland and Arnon nutrient solution was used. Then 25 mol m^{-3} NaCl was added in daily increments until final concentrations of 50, 100, 150 and 200 mM NaCl were reached. Nutrient solutions were subsequently changed once a week. The trays were arranged in a completely randomised design with 3 replicates. Plants were grown in a glasshouse, where the temperature was maintained between 16 and 24 °C with supplementary lighting provided by high pressure sodium lamps (300 μmol m^{-2} s^{-1}) for 16 h daily.

The plants were harvested 4 weeks after the final salt concentration was reached. At harvest plants were separated into young leaf blades (two youngest fully expanded leaves together with the new emerging leaf), old leaf blades, stems (including sheath) and roots. The samples were dried at 60 °C for 1 week and ground with a pestle and mortar to a fine powder for the determination of ion concentrations.

Table 2.1 Composition of Hoagland and Arnon nutrient solutions.

Hoagland		Arnon			Fe-EDTA	
chemical	usage	chemical	stock (g/l)	usage	chemical	usage
KNO_3	5 mM	H_3BO_3	2.86	1ml/l	NaFeEDTA	1 mM
$Ca(NO_3)_2 \cdot 4H_2O$	5 mM	$MnCl_2 \cdot 4H_2O$	1.81			
$MgSO_4 \cdot 7H_2O$	2 mM	$ZnSO_4 \cdot 7H_2O$	0.22			
KH_2PO_4	1 mM	$CuSO_4 \cdot 5H_2O$	0.08			
		$HMoO_4 \cdot 4H_2O$	0.09			

2.2.1.2 Plant experiment two

Two near isogenic barley cultivars Golden Promise and Maythorpe were used in this experiment. The salt treatments, germination procedures and nutrient solutions were the same as in Experiment 1. The plants were hydroponically cultured in a growth cabinet in a completely randomised design with 3 replicates under the following conditions: a day/night temperature maintained at 24 °C / 16 °C, light intensity of 300 µmol m^{-2} s^{-1} and 16 hrs light period. The plants were harvested 4 weeks after the final salt concentration was reached. This time plants were separated into the following 6

fractions: young leaf blades (two youngest fully expanded leaf blades together with the new emerging leaf), young sheaths (the corresponding sheaths of young leaf blades), old leaf blades, old sheaths, stems and roots. The samples were dried at 60 °C for 1 week and ground with a pestle and mortar for determination of ion concentrations.

2.2.1.3 Plant experiment three

Golden Promise and Maythorpe were germinated as in Experiment 1. This experiment utilised 2 treatments of 0 mM and 150 mM NaCl and 5 replicates. The plants were cultured in a growth cabinet under the following conditions: day/night temperatures of 20 °C / 10 °C, light intensity of 300 μmol m^{-2} s^{-1} and 16 hr light period. Following 3 weeks growth in a saline medium plants were transferred to a growth cabinet with similar growth conditions at IACR-Rothamsted Experimental Station. The plants were maintained for a further 5 days prior to measuring the cellular distributions of Na^+, Cl^- and K^+ using an energy-dispersive X-ray microanalysis system (EDXMA).

2.2.2 Analytical Methods

2.2.2.1 Na^+, K^+, Ca^{2+} Mg^{2+} and P determination

The total Na^+, K^+, Ca^{2+} Mg^{2+} and P concentrations of oven dried and ground tissue samples were carried out according to the method of Zhao *et al.* (1994). Approximately 0.2 g of sample was placed in a test tube with 5 ml of a nitric acid : perchloric acid mixture (85 % : 15 % v/v) and allowed to stand at room temperature for 2 hrs. Digestion was carried out in a Carbolite aluminium heating block connected to a Eurotherm 818 controller/programmer. Ramp rates, dwell times and temperatures are given in Table 2.2. After digestion and cooling to room temperature, 5 ml of 20 % HCl (v/v) was added. The tubes were mixed and rewarmed at 80 °C for 30 min. After

cooling deionised water was added to near the 20 ml mark and the tubes were rewarmed at 80 °C for a further 30 min. Upon cooling, the solution was made up to exactly 20 ml. Samples were stored at room temperature until required for analysis.

Table 2.2 Program for digestion of plant tissue for total Na^+, K^+, Ca^{2+} Mg^{2+} and P determination.

	Ramp rate (°C hr^{-1})	Dwell temp (°C)	Dwell time (hrs)
1	60	60	3
2	120	100	1
3	120	120	1
4	50	190	2

Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used to determine Na^+, K^+, Ca^{2+} Mg^{2+} and P concentrations formed by the above digestion. The measurement was carried out on a Thermo Jarrell Ash Iris Advantage ICP-AES system with a Thermo CID detector. The system was controlled by a PC running Thermo Spec/CID for Windows95 and calibrated using ICP standards (Fischer chemicals). External Certified Reference standards were used to assess the efficiency of the process.

2.2.2.2 Cl^- determination

The concentration of chloride in the tissues of the cultivars Golden Promise and Maythorpe was determined by using ion chromatography. Approximately 0.20 g (weighed accurately) of dried and ground tissue was shaken at room temperature for 2

hr in 40 ml deionised water. The extracted solution was filtered through Whatman No 1 filter paper and the extracts were either analysed the same day or stored at –20 °C.

Samples were analysed using a Dionex DX100 ion chromatogram fitted with an AS14 column, AG14 guard column (Dionex Corp.) and an AS40 autosampler. Data acquisition was carried out on a PC running Dionex Peaknet software. Sulphuric acid regenerant at 25 mM and eluant (3.6 mM sodium carbonate / 1mM sodium bicarbonate) were used. Chloride ion chromatography standards (Fischer chemicals) were used to identify and quantify peaks and to ensure that peak area was proportional to concentration over the measured range.

2.2.2.3 Na^+, Cl^- and K^+ cellular distribution

EDXMA was used to investigate Na^+, Cl^- and K^+ distributions in different cells of the youngest fully expanded leaf according to the method of Küpper *et al.* (1999). The plants were transferred from growth cabinet to laboratory immediately prior to analysis. A small middle section of the leaf was excised, mounted in a stainless steel stub, and rapidly frozen in liquid nitrogen slush. The sample was then transferred to a preparation chamber (which was cooled at -180 °C with liquid nitrogen) and fractured with a liquid nitrogen-cooled scalpel blade just above the level of the stub to reveal the surface of the cells. Ice was removed from the cell surface by exposing the sample to a high vacuum at -85 °C for 2 min. After this etching process, the sample was recooled to -180 °C and evaporatively coated with carbon to produce an electrically conductive surface. Carbon was used instead of a metal coating to avoid interference with the elements to be measured. The specimen was then transferred to a liquid-nitrogen-cooled stage (-180 °C) inside of the scanning electron microscope (SEM) (model XL

40, Philips, Eindhoven, The Netherlands). EDXMA was performed in the SEM using an acceleration voltage of 30 kV, a takeoff angle of 45°, and a working distance (sample to final lens of the SEM) of 10 mm. Spectra from 0 to 30 keV were collected at increments of 10 eV per channel with the electron beam focused on a rectangular area in the centre of selected cells. The background and element-specific peak spectra were analyzed using the program Superquant (EDAX, San Francisco, CA, USA), which fully deconvolutes the spectra and allows corrections for interference between elements. Epidermal and mesophyll cells were randomly selected for analysis of element-specific spectra and 5–10 readings were collected for each type of cell. To remove the effects of variation of surface topography between the different selected cells on the efficiency of EDXMA rate counting (peak minus background) of the elements, the oxygen signal was used to avoid shaded areas. Quantitative data were obtained using a peak/background ratio method for determination of Na^+, Cl^- and K^+ in cells with oxygen used as an internal standard (Küpper *et al.*, 2000) because oxygen appeared to be relatively homogenous in cells. The formulae for calculation of the concentrations of Cl^-, Na^+ and K^+ were supplied by IACR-Rothamsted Experimental Station.

$$Cl^- \text{ concentration } (mM) = Cl^- \text{ counts } (cps) \div O^{2-} \text{ counts } (cps) \times 1000 \div 0.6678 - 0.7373$$

$$K^+ \text{ concentration } (mM) = (K^+ \text{ counts } (cps) \div O^{2-} \text{ counts } (cps) \times 1000 - 0.37) \div 0.58$$

2.2.2.4 Chlorophyll determination

Chlorophyll concentrations of the youngest fully expanded leaves were determined indirectly using a Minolta Spad 502 meter (Japan) at week 2 and week 4 after the addition of salt to the growth medium during 9:00 to 11:00 in the morning.

2.2.2.5 Statistical analysis

Data was analysed statistically by ANOVA using the MINITAB statistical software package version 12.0.

2.3 Results

2.3.1 Experiment one

2.3.1.1 Plant growth

Golden Promise and Maythorpe showed different growth both under control (Fig. 2.1) and saline conditions (Fig. 2.2). Under control conditions, Golden Promise showed short and erect shoots and Maythorpe showed tall and collapsing shoots. Golden Promise maintained more vigorous shoot growth when compared to Maythorpe in response to 150 mM NaCl. High salinity also caused marked reductions in root growth of both Maythorpe (Fig. 2.3) and Golden Promise (Fig. 2.4). Generally, the relatively salt tolerant Golden Promise had a smaller root system than Maythorpe in response to 150 mM NaCl (Fig. 2.5). Photographs of Claret and Clansman are not presented here but the growth habits of the two *GPert* mutants Golden Promise and Clansman were very similar in the absence of and presence of salt. The two non-*GPert* cultivars Maythorpe and Claret exhibited similar growth under both control and saline conditions although Maythorpe exhibited more vigorous growth than Claret.

Fig. 2.1 Growth of the shoots of Maythorpe (right) and Golden Promise (left) following 4 weeks growth in the absence of NaCl (control).

Fig. 2.2 Growth of the shoots of Maythorpe (right) and Golden Promise (left) following 4 weeks growth in 150 mM NaCl.

Fig. 2.3 Growth of the roots of Maythorpe under normal nutrition (left) and 150 mM NaCl (right) for 4 weeks.

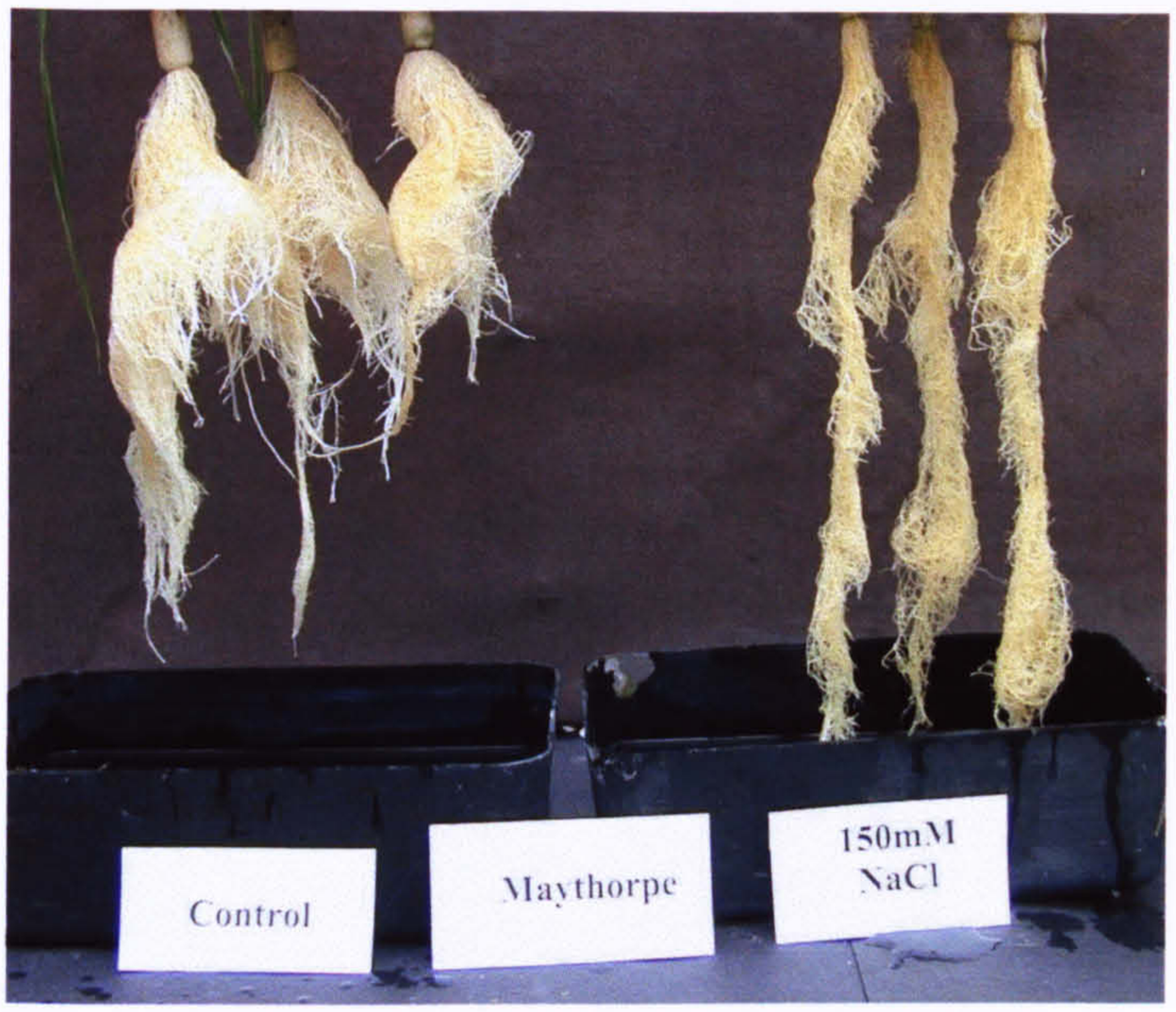


Fig. 2.4 Growth of the roots of Golden Promise under normal nutrition (left) and in the presence of 150 mM NaCl (right) for 4 weeks.

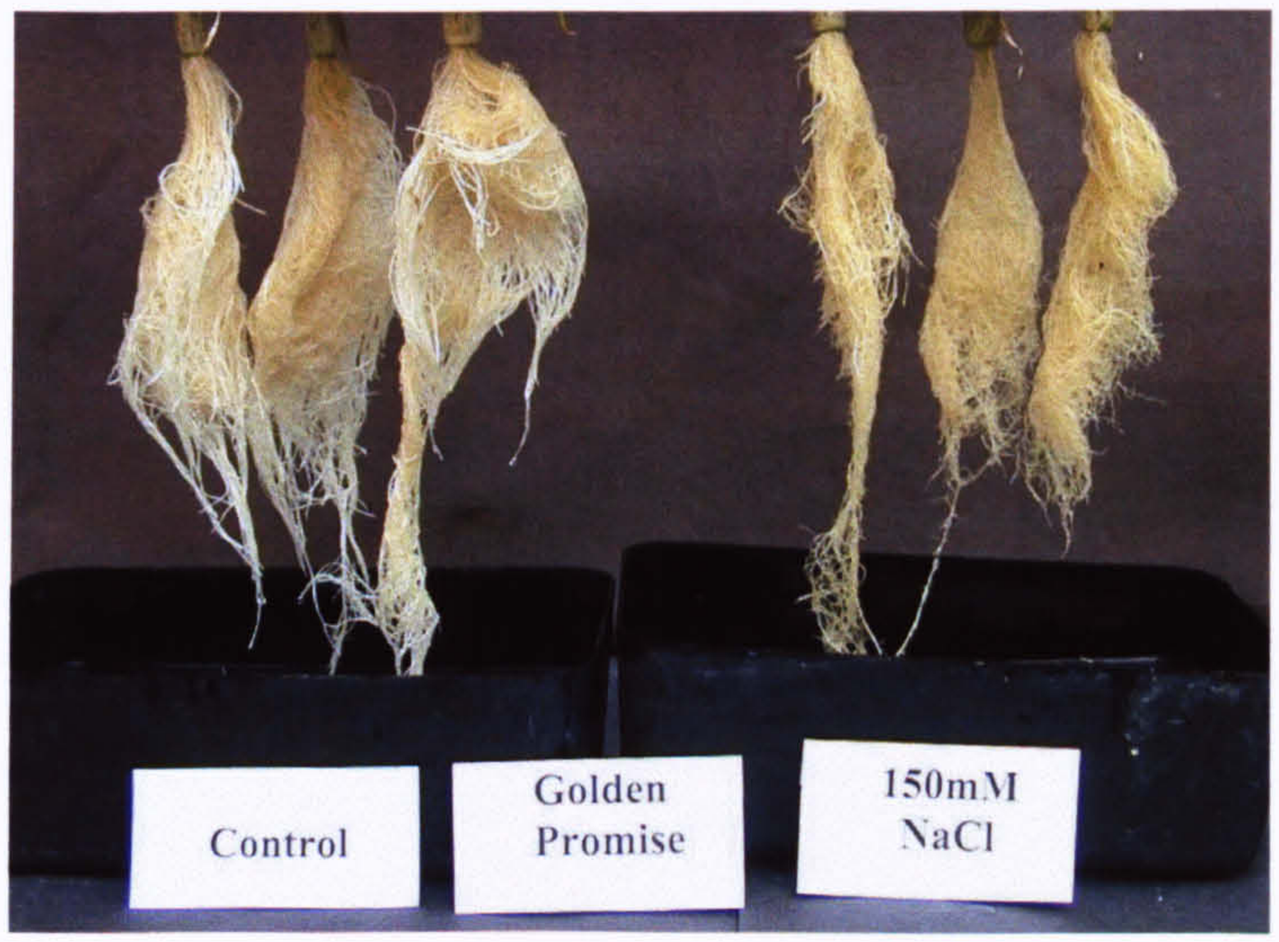
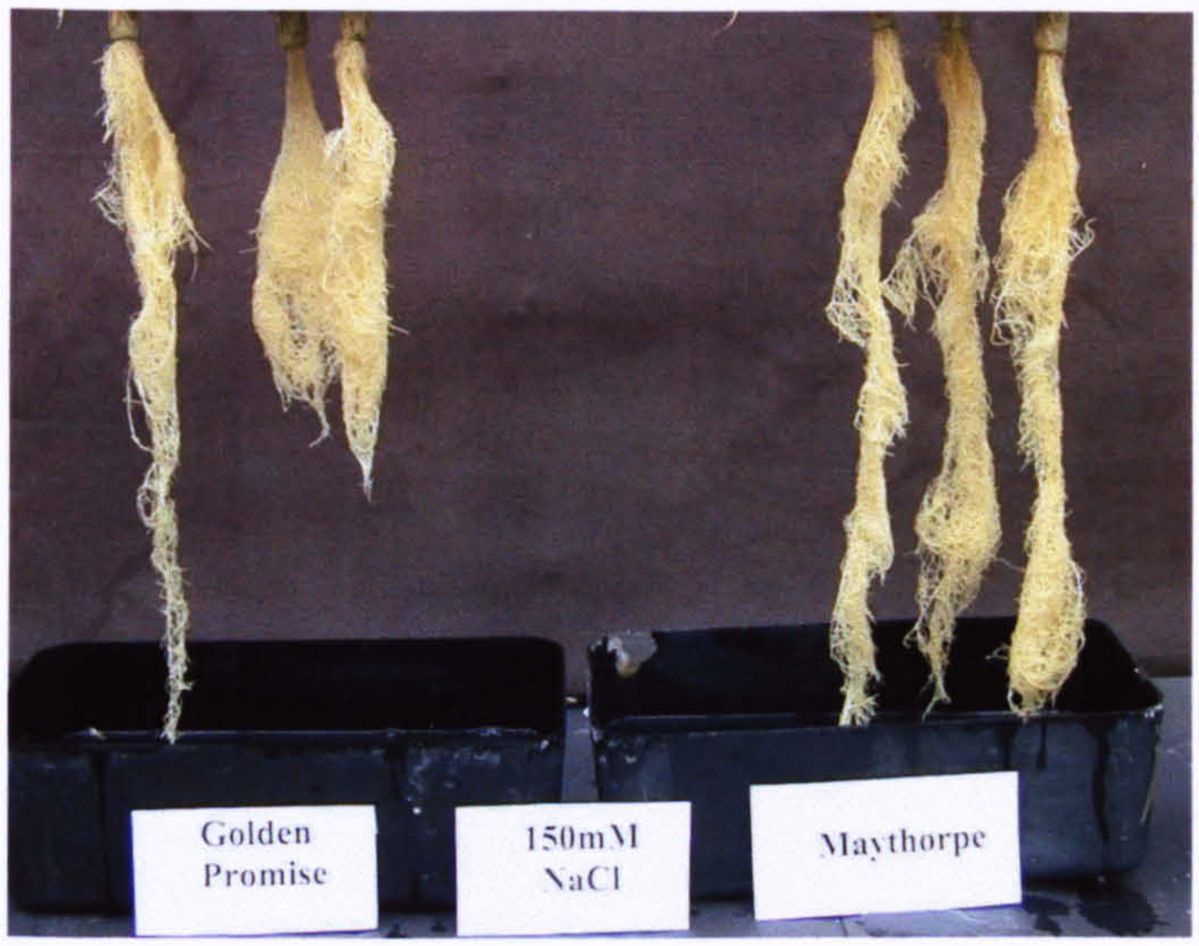


Fig. 2.5 Growth of the roots of Maythorpe (right) and Golden Promise (left) following 4 weeks growth in 150 mM NaCl.



Shoot fresh and dry weight of Maythorpe, Golden Promise, Clansman and Claret were all significantly reduced ($p<0.01$) with increasing NaCl in the growth medium (Table 2.3). The greatest reduction in growth of all cultivars was in response to the first 50 mM of NaCl. There were no significant differences in both fresh and dry weight of individual cultivars beyond 100 mM NaCl. Maythorpe produced more shoot fresh and dry matter than Golden Promise and Claret produced more than Clansman across all the treatments. Reductions in root growth in the presence of salt were greater in the non-*GPert* cultivars Maythorpe and Claret than *GPert* cultivars Golden Promise and Clansman (Table 2.4). The difference in the response to salinity between the cultivars can be seen when dry weight is expressed in terms of comparative growth of shoot

(CGS) and root (CGR), *i.e.* the growth of each treatment as a percentage of the corresponding control (Table 2.5). Golden Promise and Clansman maintained higher CGS than Maythorpe and Claret, respectively, especially under high salinity. For example, Golden promise had higher CGS than Maythorpe and Clansman had higher CGS than Claret from 150 to 200 mM NaCl. Golden Promise and Clansman maintained consistently higher CGR than Maythorpe and Claret, respectively throughout all salt treatments. Compared with CGS and CGR, increasing salinity showed different effects on the growth of shoot and root of the cultivars. In the two comparatively salt sensitive cultivars Maythorpe and Claret, the root growth was even more affected by the increasing salinity. In contrast, in the two comparatively salt tolerant cultivars Golden Promise and Clansman the root growth was less affected by the increasing salt concentrations in the growth medium.

Increasing salinity caused a reduction in the ratio of young leaf blade to old leaf blade (YLB:OLB) of all cultivars (Table 2.6), but overall, there were no obvious varietal differences either between Maythorpe and Golden Promise or Claret and Clansman. However at low salinity *i.e.* 50 to 100 mM NaCl, Golden Promise maintained slightly higher YLB:OLB ratios than Maythorpe.

Table 2.3 Shoot dry and fresh weight (g/plant) of barley cultivars Maythorpe, Golden Promise, Clansman and Claret growing 4 weeks under 0, 50, 100, 150 and 200 mM NaCl after the final concentration of 200 mM NaCl reached. Values are means (±se) of 3 replicates.

NaCl (mM)	Shoot dry weight				Shoot fresh weight			
	Maythorpe	Golden Promise	Claret	Clansman	Maythorpe	Golden Promise	Claret	Clansman
0	8.16±1.17 a	4.35±0.15 a	5.89±0.84 a	4.36±0.42 a	66.63±5.22 a	43.52±3.76 a	43.83±4.89 a	37.30±2.02 a
50	4.84±0.26 b	2.67±0.41 bc	3.72±0.60 ab	2.80±0.57 ab	41.83±0.55 b	22.52±2.76 b	27.20±3.41 b	19.72±1.78 b
100	2.74±0.14 c	1.67±0.17 cd	2.17±0.21 bc	1.54±0.10 bc	20.95±5.24 c	11.55±1.65 bc	13.60±1.01 bc	9.93±0.43 c
150	2.11±0.16 c	1.30±0.12 d	1.28±0.32 bc	1.09±0.12 c	13.88±0.48 c	8.63±0.54 c	7.47±1.61 c	6.11±0.55 c
200	1.59±0.05 c	1.01±0.03 d	1.02±0.12 c	0.91±0.11 c	9.67±0.40 c	6.08±0.02 c	5.58±0.71 c	5.95±0.70 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

Table 2.4 Root dry weight (g/plant) of barley cultivars Maythorpe, Golden Promise, Clansman and Claret growing 4 weeks under 0, 50, 100, 150 and 200 mM NaCl after the final concentration of 200 mM NaCl reached. Values are means (±se) of 3 replicates.

NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
0	1.45±0.09 a	0.62±0.02 a	1.13±0.18 a	0.64±0.05 a
50	0.92±0.08 b	0.56±0.09 ab	0.64±0.22 ab	0.50±0.05 a
100	0.48±0.02 c	0.32±0.04 bc	0.39±0.07 b	0.26±0.04 b
150	0.45±0.10 c	0.29±0.04 c	0.17±0.05 b	0.15±0.02 b
200	0.30±0.04 c	0.22±0.03 c	0.15±0.02 b	0.14±0.02 b

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

Table 2.5 Comparative growth of shoot (CGS) and root (CGR) of barley cultivars Maythorpe, Golden Promise, Clansman and Claret growing 4 weeks under 0, 50, 100, 150 and 200 mM NaCl after the final concentration of 200 mM NaCl reached.

NaCl (mM)	Maythorpe		Golden Promise		Claret		Clansman	
	CGS	CGR	CGS	CGR	CGS	CGR	CGS	CGR
	%							
0	100	100	100	100	100	100	100	100
50	65.41	63.40	61.46	90.32	63.16	56.64	64.12	78.13
100	37.62	33.10	38.26	51.61	36.76	34.51	35.36	40.63
150	28.50	31.03	29.80	46.77	21.73	15.04	24.90	23.44
200	21.58	20.69	23.33	35.48	17.29	13.27	20.80	21.88

* Comparative growth: CGS = shoot dry weight of salt treatment / shoot dry weight of control × 100; CGR = root dry weight of treatment / root dry weight of control treatment × 100.

Table 2.6 YLB:OLB ratios of barley cultivars in relation to 0, 50, 100, 150 and 200 mM NaCl.

NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
0	1.18±0.14 a	1.18±0.20 ab	1.20±0.08 a	1.39±0.10 a
50	1.16±0.06 a	1.30±0.05 a	1.24±0.27 a	1.17±0.10 ab
100	1.07±0.41 .a	1.16±0.03 ab	0.95±0.14 a	0.92±0.01 abc
150	1.08±0.21 a	1.04±0.03 ab	0.71±0.06 a	0.64±0.07 c
200	0.96±0.08 a	0.80±0.04 b	0.71±0.06 a	0.80±0.14 bc

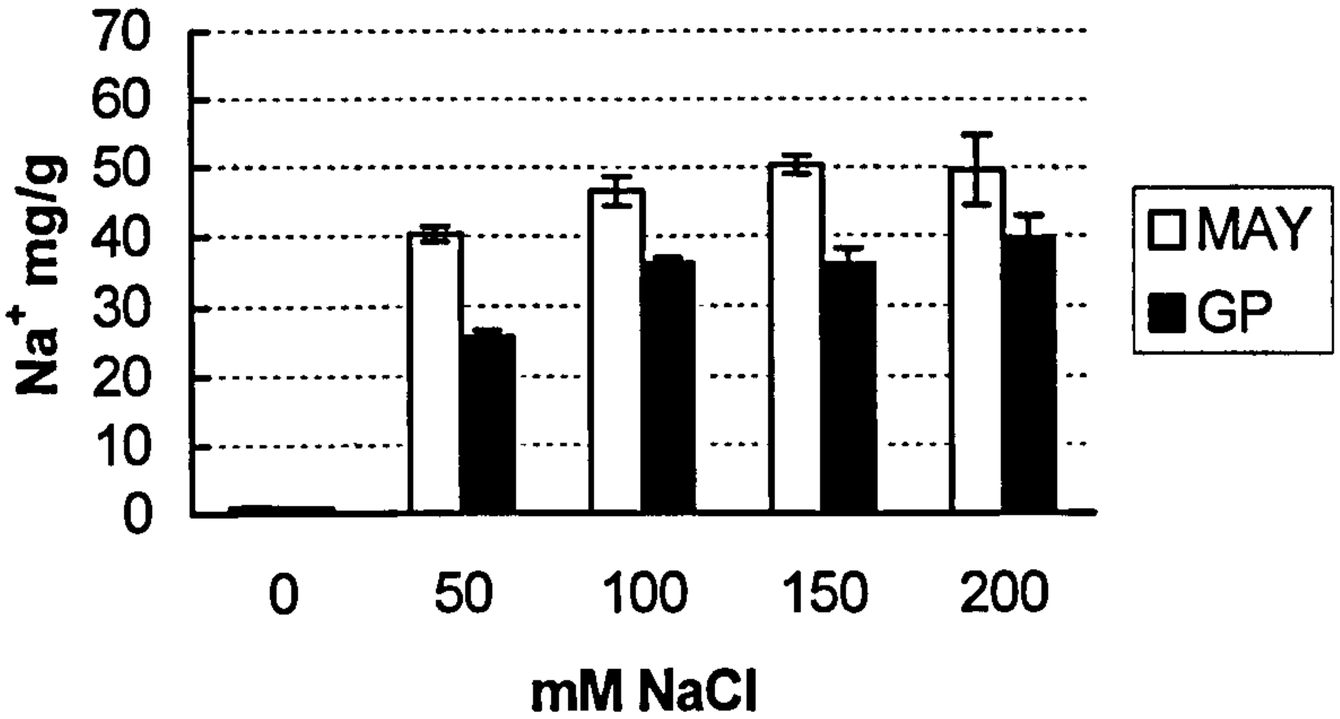
* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.2 Na^+ concentrations

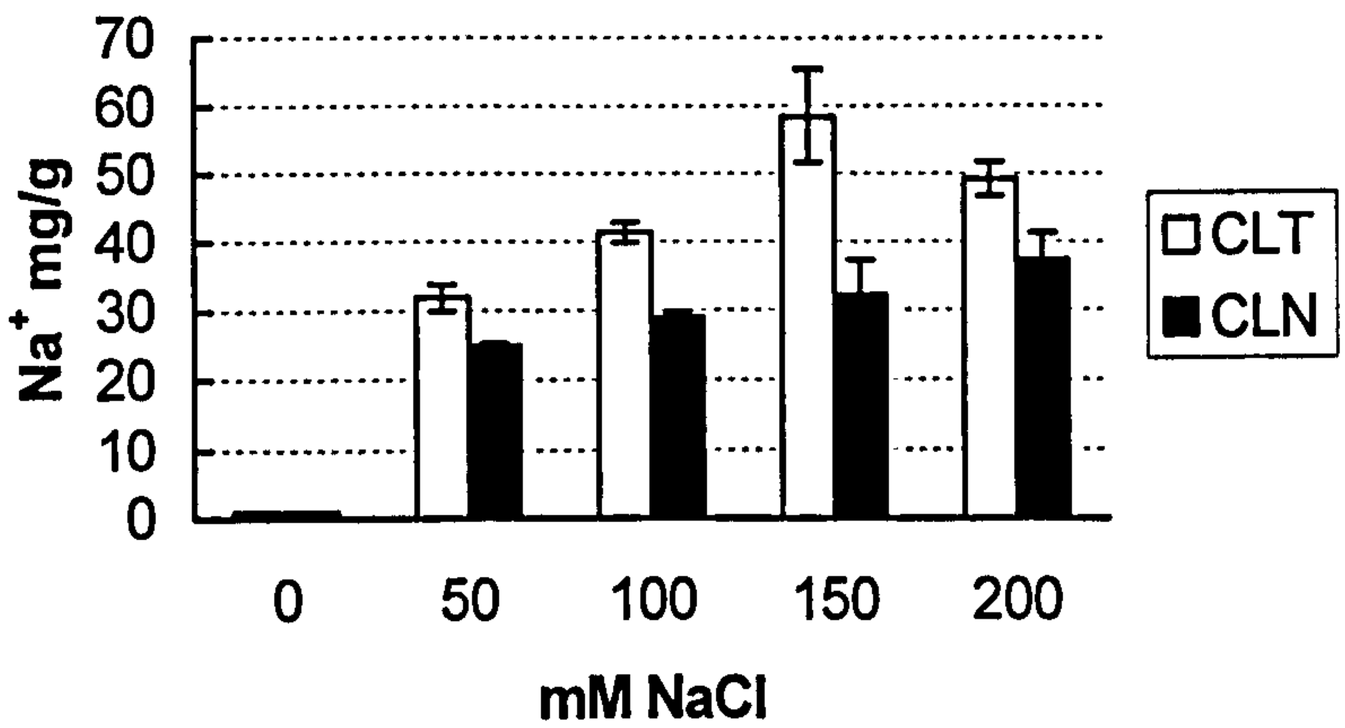
Na^+ concentrations are presented in terms of whole plant Na^+ concentration and individual tissue Na^+ concentration. Whole plant Na^+ concentrations of the four cultivars increased in response to increasing salinity (Fig. 2.6). The two relatively salt tolerant cultivars Golden Promise and Clansman contained significantly lower ($p<0.01$) Na^+ than Maythorpe and Claret, respectively. At 50, 100, 150 and 200mM NaCl, Golden Promise contained 36.3 %, 22.3 %, 28.2 % and 19.8 % lower Na^+ than Maythorpe, and Clansman contained 21.5 %, 30.1 %, 45.0 % and 24.0 % lower Na^+ than Claret, respectively.

Fig. 2.6 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and plant Na^+ concentrations (mg/g dry weight, means ± se). a: Maythorpe (MAY) and Golden Promise (GP); b: Claret (CLT) and Clansman (CLN).

a



b



The differences in Na^+ concentrations between cultivars were also evident on an individual tissue basis (Table 2.7). Golden Promise contained significantly lower ($p<0.01$) Na^+ than Maythorpe in all tissues. Clansman also contained significantly lower Na^+ than Claret in young leaf blade ($p<0.01$), old leaf blade ($p<0.01$), stem ($p<0.01$) and root ($p<0.05$) tissues. The Na^+ concentrations of all tissues were increased in the cultivars in response to increasing salinity with the largest increase occurring between 0 and 50 mM NaCl. The Na^+ concentrations of young leaf blade, old leaf blade and stem tissues of Golden Promise and Maythorpe were largely unchanged at high NaCl concentrations from 150 to 200 mM with the exception of the stem of Golden Promise. The highest Na^+ concentrations normally occurred at 100 or 150 mM NaCl in the above shoot tissues. Like Golden Promise, the Na^+ concentrations of young leaf blade and stem of Clansman showed little change from 100 to 200 mM NaCl, but in the old leaf blade the Na^+ concentration raised with increasing external NaCl although the difference was not significant. In Claret, the Na^+ concentration in the young leaf blade increased about 22 % as the NaCl concentration in the growth media was raised from 150 to 200 mM NaCl, but in the old leaf blade and stem tissues the highest Na^+ concentrations occurred at 150 mM NaCl. In the root tissue of all cultivars the Na^+ concentrations were increased with increasing external NaCl, the highest Na^+ concentrations occurred at 200 mM NaCl. The differences in Na^+ concentrations between Clansman and Claret were generally greater than those between Golden Promise and Maythorpe with increasing salinity from 100 to 200 mM NaCl. In general, the lowest Na^+ containing tissue was young leaf blades and the highest was old leaf blades in all 4 cultivars under salt stress with the exception of the treatment of 200 mM NaCl where the highest Na^+ concentration occurred in the roots of Maythorpe, Golden Promise and Claret.

Table 2.7 Na^+ concentrations (mg/g dry weight) in individual tissues of Maythorpe, Golden Promise, Clansman and Claret in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	0	0.69±0.09 a	0.55±0.05 a	0.69±0.08 a	0.71±0.02 a
	50	24.36±1.09 b	15.35±1.11 b	18.85±4.49 b	13.91±1.31 b
	100	28.38±2.85 b	22.73±1.27 b	25.40±1.17 bc	15.82±0.83 b
	150	29.84±2.19 b	20.11±2.28 b	27.98±4.12 bc	14.22±2.63 b
	200	28.37±4.80 b	20.84±1.96 b	34.06±3.78 c	14.12±1.03 b
Old leaf blade	0	0.88±0.16 a	0.64±0.02 a	0.88±0.10 a	1.00±0.04 a
	50	49.15±1.71 b	30.57±1.48 b	40.70±5.70 b	34.10±0.75 ab
	100	63.65±5.70 b	44.31±0.99 c	60.44±2.47 bc	32.36±4.21 ab
	150	59.14±6.02 b	43.96±3.69 c	83.39±13.83 bc	50.74±12.32 b
	200	57.41±5.59 b	42.64±2.59 c	66.11±2.67 c	61.65±11.74 b
Stem	0	0.92±0.16 a	0.69±0.05 a	0.89±0.11 a	0.94±0.03 a
	50	46.19±3.39 b	29.97±1.18 b	33.92±3.45 b	24.25±0.81 b
	100	46.98±5.02 b	40.90±3.74 c	38.78±2.90 bc	28.56±1.35 b
	150	52.31±4.99 b	37.84±0.77 c	48.83±3.06 bc	27.88±3.52 b
	200	51.68±9.51 b	41.90±2.83 c	39.90±0.81 c	30.11±1.48 b
Root	0	1.31±0.46 a	1.22±0.14 a	1.34±0.15 a	1.71±0.07 a
	50	40.33±3.62 b	26.55±1.31 b	30.05±4.67 b	30.58±1.28 b
	100	51.73±2.34 b	35.74±0.90 bc	44.84±4.85 bc	40.47±0.61 bc
	150	57.52±2.09 b	42.28±3.13 bc	60.15±5.28 c	37.04±1.90 b
	200	59.45±10.45 b	50.70±7.90 c	68.81±7.84 c	56.67±8.31 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.3 Cl^- concentrations

Chloride concentrations in both Golden Promise and Maythorpe were closely related to external Cl^- concentrations (Fig. 2.7). With each increment of salt concentration in the growth media causing an increase in whole plant Cl^-. No significant difference was observed between the two cultivars. Similar results can also be seen for individual tissues (Table 2.8), where each increment of salt from 0 to 200 mM NaCl resulted in an increased Cl^- concentration of all tissues. There was also no significant varietal difference in individual tissue concentrations. Under low salinity, *i.e.* 50 mM NaCl, the lowest Cl^- concentrations was found in young leaf blades and the highest in stem tissue, but under high salinity, *i.e.* 150 and 200 mM NaCl, the lowest Cl^- concentration occurred in roots and the highest was in old leaf blades in both cultivars.

Fig. 2.7 Relationship between plant Cl^- concentrations (mg/g dry weight, means ± se) and salt treatments of 0, 50, 100, 150 and 200 mM NaCl in Golden Promise and Maythorpe.

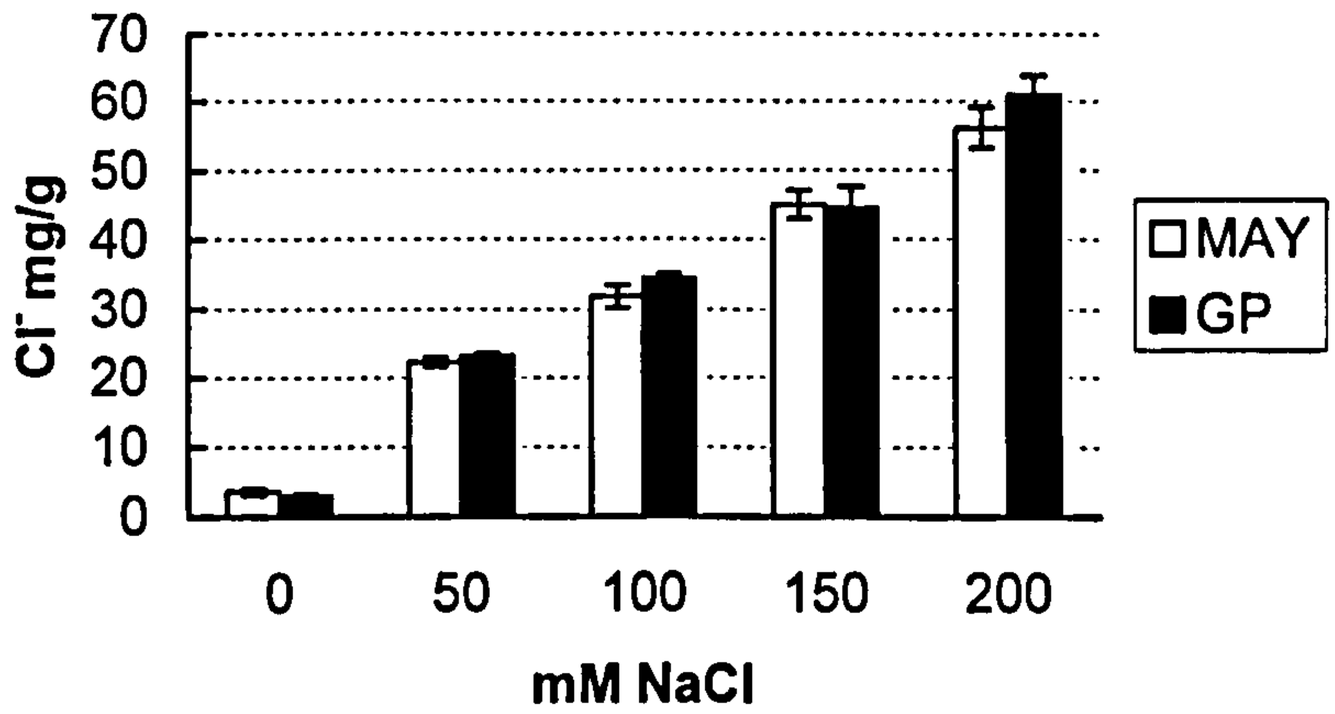


Table 2.8 Cl^- concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	2.43±0.19 a	2.28±0.44 a
	50	14.27±0.77 ab	14.95±1.36 b
	100	19.64±1.40 b	22.75±0.98 bc
	150	27.80±0.61 bc	28.36±1.34 cd
	200	37.01±5.89 c	38.43±3.74 d
Old leaf blade	0	3.48±0.74 a	2.76±0.42 a
	50	25.70±0.75 b	27.72±1.57 b
	100	46.67±0.87 c	48.13±1.64 c
	150	67.80±2.45 d	68.93±6.30 d
	200	95.36±4.42 e	94.88±4.64 e
Stem	0	5.58±0.76 a	4.44±0.51 a
	50	29.54±0.97 b	30.31±0.68 b
	100	38.09±2.95 bc	42.71±2.11 c
	150	51.03±3.64 cd	49.55±2.46 c
	200	54.17±4.99 d	64.52±3.56 d
Root	0	1.25±0.20 a	1.15±0.11 a
	50	15.60±2.97 ab	15.70±2.69 ab
	100	20.67±5.29 ab	18.16±2.38 ab
	150	25.14±6.70 b	24.23±5.55 ab
	200	28.34±3.10 b	28.58±8.15 b

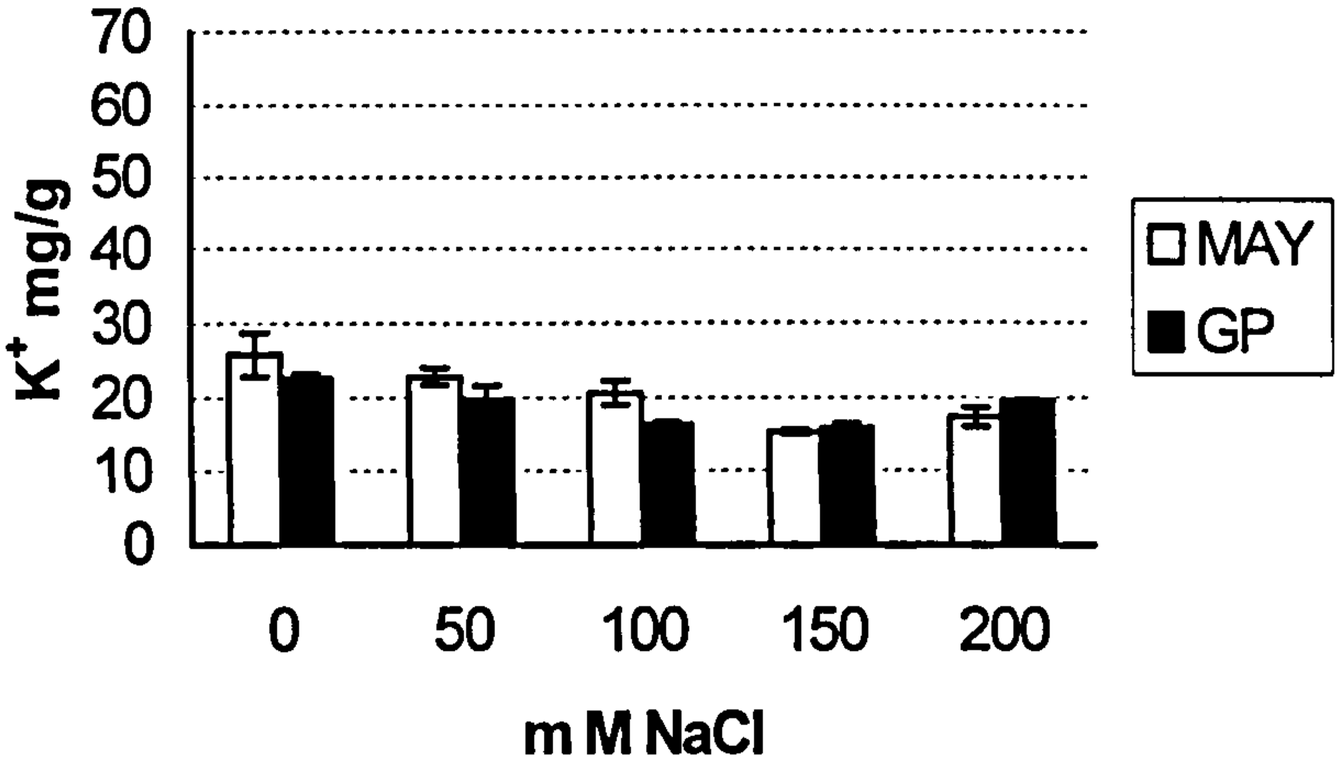
* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.4 K^+ concentrations and K^+/Na^+ ratios

Increasing the salt concentration up to 100 mM NaCl resulted in a reduction of K^+ concentrations of the plants, beyond which there was no marked effect (Fig. 2.8). Claret and Clansman both contained higher K^+ concentrations than Golden Promise and Maythorpe under control conditions. No significant differences in K^+ concentrations were observed between Golden Promise and Maythorpe and between Clansman and Claret.

Fig. 2.8 Relationship between whole plant K^+ concentrations (mg/g dry weight, means ± se) and salt treatments of 0, 50, 100, 150 and 200 mM NaCl. a: Maythorpe (MAY) and Golden Promise (GP); b: Claret (CLT) and Clansman (CLN).

a



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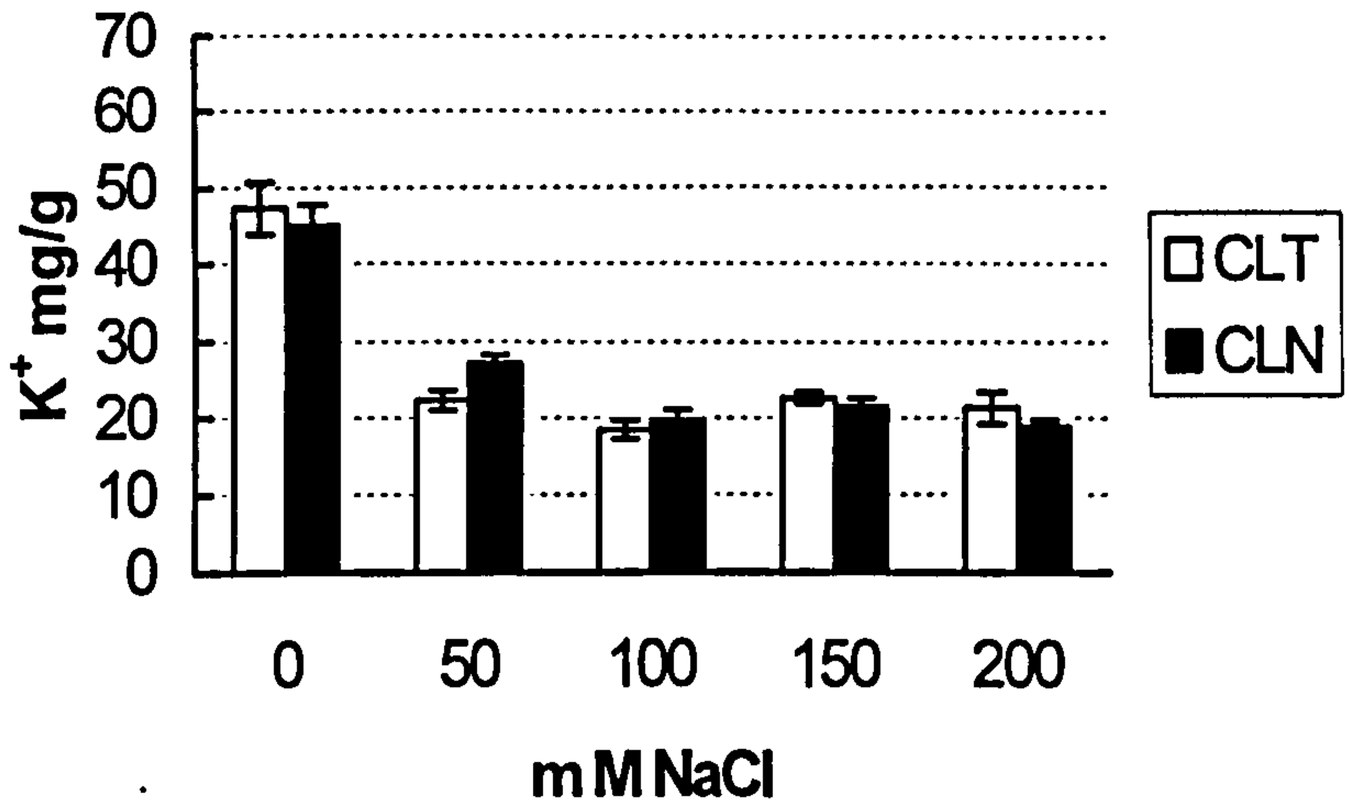


When the plants were separated into individual tissues, there were also no significant differences between Golden Promise and Maythorpe and between Clansman and Claret in K^+ concentrations (Table 2.9). No significant effect of increasing salinity on K^+ concentrations was observed in individual tissues of Maythorpe and Golden Promise with the exception of the old leaf blade of Golden Promise where 50 and 100 mM NaCl resulted in significant reductions in K^+ concentration. But in Claret and Clansman, salinity resulted in significant reductions in K^+ concentration in old leaf blade, stem and root tissues compared to the corresponding controls. However, K^+/Na^+ ratios showed varietal differences between Golden Promise and Maythorpe and between Clansman and Claret in response to salinity (Table 2.10). In the presence of salt Golden Promise maintained significantly higher K^+/Na^+ ratios in young leaf blade ($p<0.01$), old leaf blade ($p<0.01$), stem ($p<0.01$) and root ($p<0.05$) than the corresponding tissues of Maythorpe; while Clansman maintained significantly higher K^+/Na^+ ratios in young leaf blade ($p<0.01$), old leaf blade ($p<0.01$), and stem ($p<0.01$) than the corresponding tissues of Claret.

Table 2.9 K^+ concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	0	21.57±3.54 a	16.67±1.99 a	35.75±4.69 a	39.31±3.13 a
	50	21.86±1.42 a	22.45±4.18 a	19.99±2.03 a	31.09±5.17 ab
	100	23.36±4.74 a	18.62±0.33 a	18.04±1.31 a	24.25±2.14 ab
	150	17.64±0.93 a	18.48±0.99 a	24.65±2.01 a	23.18±2.26 b
	200	20.41±1.25 a	23.47±1.85 a	25.63±7.80 a	22.64±2.02 b
Old leaf blade	0	18.07±4.48 a	18.67±2.06 a	37.50±4.73 a	40.54±2.05 a
	50	18.47±0.51 a	11.28±0.45 b	10.70±1.39 d	13.21±0.85 b
	100	14.34±0.54 a	10. 20±0.57 b	13.24±0.12 cd	11.61±2.49 b
	150	13.06±0.68 a	13.89±0.87 ab	23.18±1.57 bc	19.96±2.54 b
	200	15.88±0.51 a	18.60±1.57 a	21.43±0.31 cd	18.29±1.56 b
Stem	0	21.17±5.51 a	24.48±1.89 a	54.25±1.55 a	51.14±4.09 a
	50	26.27±2.59 a	22.61±2.73 a	26.14±2.56 b	29.93±0.97 b
	100	23.66±2.20 a	19.42±0.30 a	22.11±2.15 b	21.86±1.46 b
	150	17.24±0.77 a	17.93±0.72 a	23.76±2.23 b	24.29±1.23 b
	200	19.85±2.76 a	23.05±0.71 a	22.90±2.13 b	20.84±0.85 b
Root	0	17.42±5.71 a	17.40±1.92 ab	48.89±6.40 a	40.07±2.07 a
	50	22.63±1.33 a	19.06±2.78 a	25.84±0.58 b	27.37±1.16 b
	100	18.32±2.72 a	14.26±0.71 ab	12.87±0.76 bc	16.87±0.62 c
	150	10.96±0.93 a	11.17±0.11 ab	12.13±1.30 bc	9.69±0.90 d
	200	9.31±1.55 a	8.97±2.31 b	10.01±1.45 c	7.25±0.66 d

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

Table 2.10 K^+/Na^+ ratios of individual tissues in response to 50, 100, 150 and 200 mM NaCl. Values are means of 3 replicates.

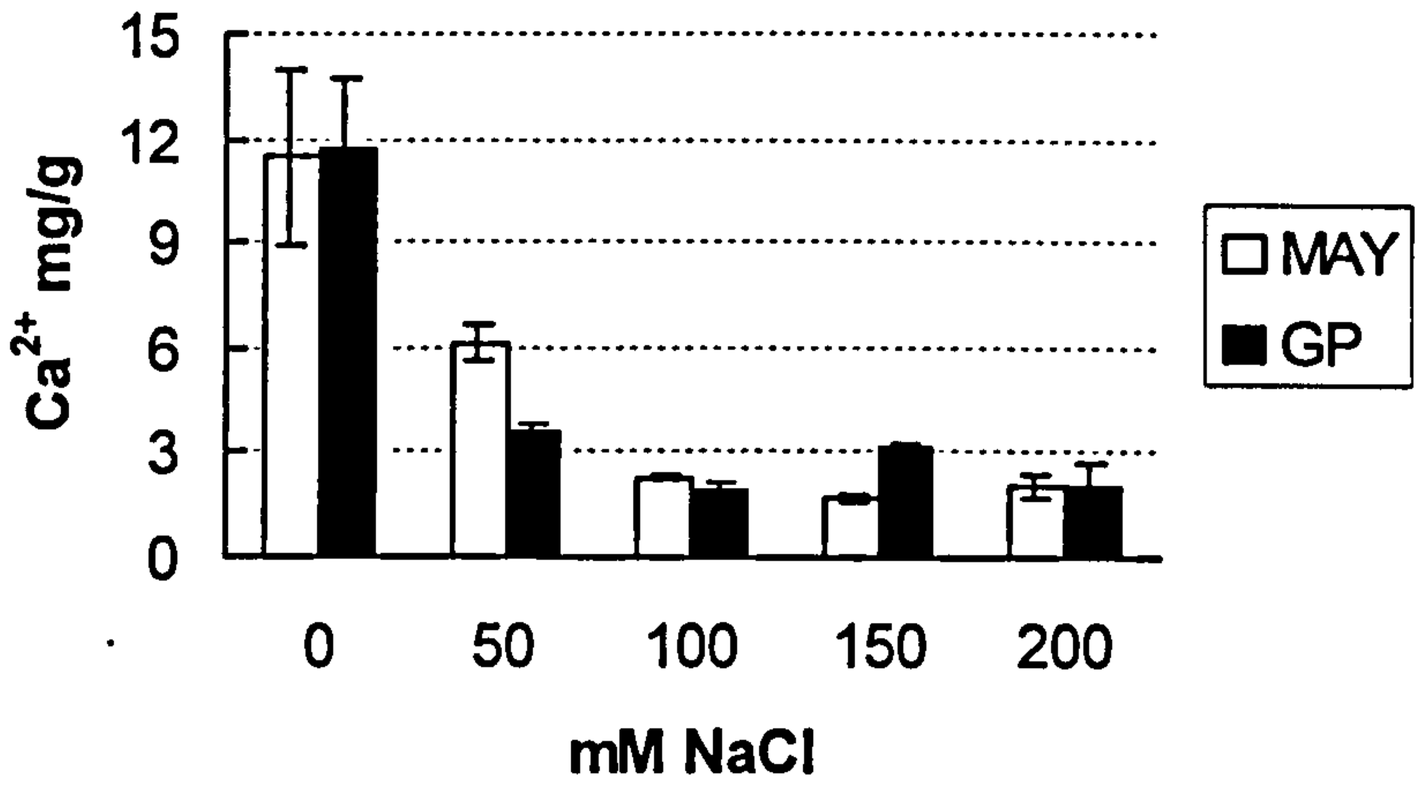
Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	50	0.91 a	1.46 a	1.13 a	2.20 a
	100	0.84 a	0.82 a	0.71 a	1.54 a
	150	0.59 a	0.92 a	0.88 a	1.69 a
	200	0.70 a	1.16 a	0.72 a	1.61 a
Old leaf blade	50	0.38 a	0.38 ab	0.26 ab	0.39 a
	100	0.23 b	0.23 a	0.22 a	0.35 a
	150	0.21 b	0.31 ab	0.29 ab	0.42 a
	200	0.28 b	0.44 b	0.33 b	0.33 a
Stem	50	0.57 a	0.75 a	0.77 a	1.24 a
	100	0.50 ab	0.48 b	0.57 ab	0.77 b
	150	0.33 c	0.47 b	0.49 b	0.90 ab
	200	0.39 bc	0.55 ab	0.58 ab	0.69 b
Root	50	0.57 a	0.71 a	0.89 a	0.90 a
	100	0.35 b	0.40 b	0.30 b	0.42 b
	150	0.19 bc	0.27 bc	0.20 b	0.26 bc
	200	0.16 c	0.17 c	0.14 b	0.13 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.5 Ca^{2+} concentrations and Ca^{2+}/Na^{+} ratios

Increasing salinity also caused a reduction in whole plant Ca^{2+} concentrations (Fig. 2.9) of all four cultivars. This reduction was greatest with increasing salinity up to 100 mM NaCl, while beyond 100 mM NaCl there was little change in any of the cultivars. No significant difference was observed between Golden Promise and Maythorpe and between Clansman and Claret in whole plant Ca^{2+} concentrations.

Fig. 2.9 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and whole plant Ca^{2+} concentrations (mg/g dry weight, means ± se). a: Maythorpe (MAY) and Golden Promise (GP); b: Claret (CLT) and Clansman (CLN).



Increasing salt concentration caused a large reduction in Ca^{2+} concentration in individual tissues with the largest decrease occurring in response to the first 50 mM NaCl supplied (Table 2.11). Generally, Ca^{2+} concentrations of individual tissues of Golden Promise and Clansman increased from 100 to150 mM NaCl while those of Maythorpe and Claret decreased over the corresponding range.

Table 2.11 Ca^{2+} concentrations (mg/g dry weight) in individual tissues of Maythorpe, Golden Promise, Clansman and Claret in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	0	6.30±0.19 a	7.23±0.30 a	5.21±0.06 a	6.24±0.45 a
	50	2.93±0.42 b	1.74±0.03 b	2.20±0.26 b	2.89±0.13 b
	100	1.39±0.14 c	1.11±0.13 b	2.15±0.16 b	1.89±0.16 b
	150	1.04±0.07 c	2.17±0.41 b	2.65±0.81 b	2.63±0.52 b
	200	1.21±0.12 c	1.70±0.64 b	2.10±0.22 b	2.11±0.09 b
Old leaf blade	0	15.83±1.02 a	17.54±1.13 a	12.70±1.75 a	16.66±2.01 a
	50	8.34±1.59 b	5.50±0.41 b	7.84±0.89 b	9.82±0.32 b
	100	4.64±0.43 bc	3.18±0.75 b	8.46±0.70 ab	5.92±1.00 b
	150	3.36±0.10 c	6.03±0.42 b	6.72±0.33 b	7.37±0.11 b
	200	3.76±0.97 bc	3.87±1.44 b	4.85±0.26 b	6.00±0.44 b
Stem	0	5.01±0.24 a	5.03±0.24 a	3.98±0.21 a	3.83±0.05 a
	50	3.02±0.56 b	1.98±0.04 b	2.29±0.28 b	1.77±0.24 b
	100	1.55±0.23 bc	1.58±0.18 bc	1.75±0.07 b	1.35±0.13 b
	150	1.23±0.22 c	1.83±0.05 bc	1.65±0.16 b	1.61±0.25 b
	200	1.54±0.25 bc	1.11±0.32 c	1.55±0.09 b	1.32±0.12 b
Root	0	29.98±6.09 a	33.64±5.65 a	21.15±5.52 a	33.06±5.38 a
	50	14.22±3.47 ab	8.15±2.13 b	8.11±0.76 b	19.76±5.07 ab
	100	2.57±0.08 b	1.98±0.38 b	4.06±0.54 b	3.40±0.24 bc
	150	1.81±0.23 b	2.77±0.35 b	2.98±0.16 b	3.09±0.18 bc
	200	1.90±0.26 b	1.57±0.37 b	2.39±0.16 b	2.42±0.18 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

Although no significant differences in Ca^{2+} concentrations of the individual tissues were detected between Golden Promise and Maythorpe and between Clansman and Claret, the Ca^{2+}/Na^{+} ratios showed varietal differences (Table 2.12). In the presence of salt, Golden Promise maintained significantly higher Ca^{2+}/Na^{+} ratios in young leaf blade ($p<0.05$), old leaf blade ($p<0.05$) and stem ($p<0.05$) than the corresponding tissues of Maythorpe, no significant difference was observed in root. Clansman maintained significantly higher Ca^{2+}/Na^{+} ratios in young leaf blade ($p < 0.01$), old leaf blade ($p < 0.01$), stem ($p < 0.05$) and root ($p < 0.01$) than the corresponding tissues of Claret. The largest decrease in Ca^{2+}/Na^{+} ratios in all cultivars was in response to the first 50 mM of NaCl.

Table 2.12 Ca^{2+}/Na^{+} ratios in individual tissues of Maythorpe, Golden Promise, Clansman and Claret in response to 50, 100, 150 and 200 mM NaCl. Values are means of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	50	0.12 a	0.12 a	0.12 a	0.21 a
	100	0.05 b	0.05 a	0.08 b	0.12 c
	150	0.04 b	0.11 a	0.09 b	0.19 ab
	200	0.04 b	0.08 a	0.06 b	0.15 bc
Old leaf blade	50	0.17 a	0.18 a	0.19 a	0.29 a
	100	0.07 b	0.07 b	0.14 b	0.18 b
	150	0.05 b	0.14 ab	0.08 c	0.16 b
	200	0.07 b	0.09 b	0.07 c	0.10 b
Stem	50	0.07 a	0.07 a	0.07 a	0.07 a
	100	0.03 a	0.04 bc	0.05 b	0.05 b
	150	0.02 a	0.07 ab	0.03 bc	0.06 ab
	200	0.03 a	0.03 c	0.04 c	0.04 b
Root	50	0.36 a	0.31 a	0.27 a	0.65 a
	100	0.05 b	0.06 b	0.09 b	0.08 b
	150	0.03 b	0.06 b	0.05 bc	0.08 b
	200	0.04 b	0.03 b	0.04 c	0.04 b

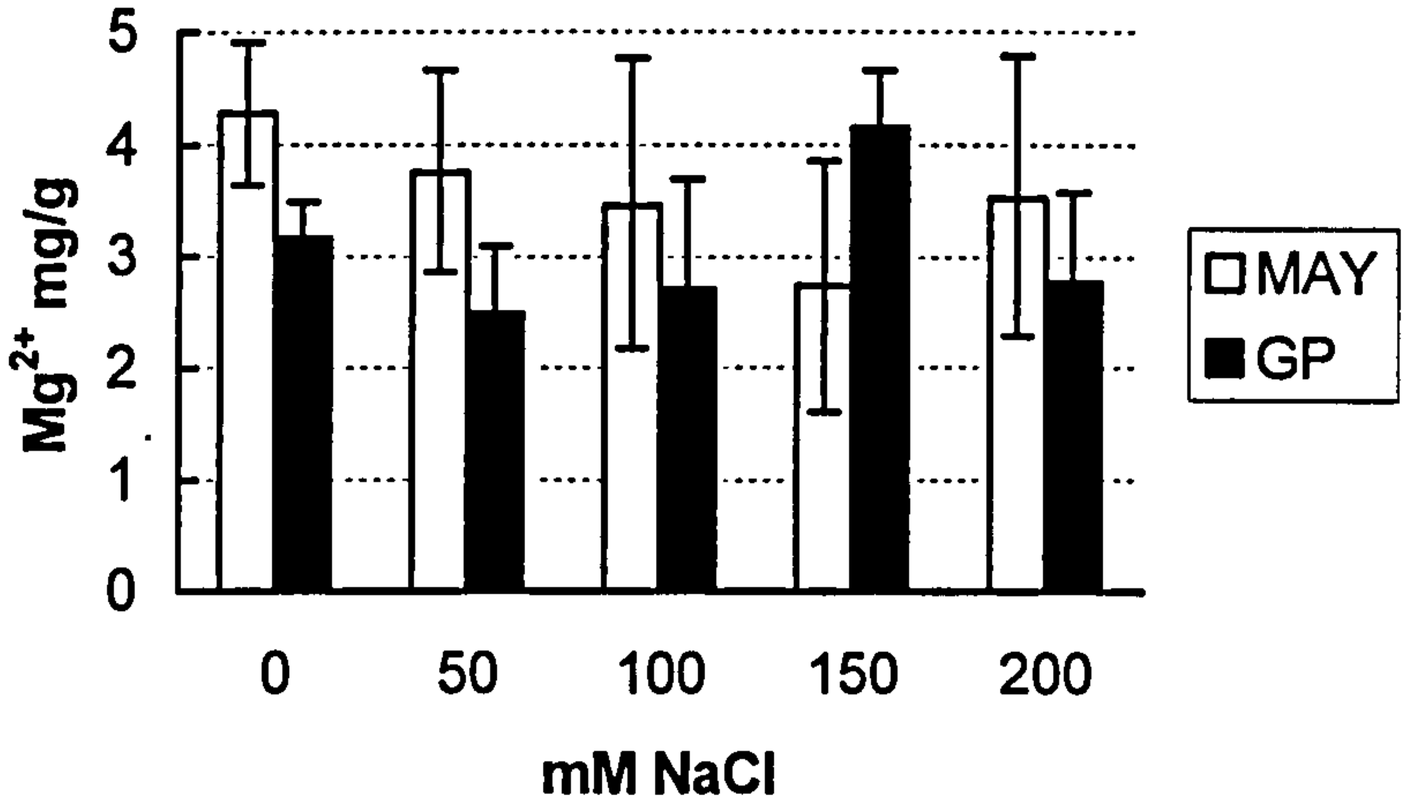
* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.6 Mg^{2+} concentrations

Maythorpe maintained higher whole plant Mg^{2+} concentrations than Golden Promise at all salinity levels with the exception of 150 mM NaCl, but the difference was not significant because of the large variability in Mg^{2+} concentration (Fig. 2.10a). There were no consistent differences between Claret and Clansman (Fig. 2.10b). In the absence of, and at low salt concentrations there was very little difference in Mg^{2+} concentrations of above ground individual tissues between Maythorpe and Golden Promise with the major difference being in root tissues (Table 2.13). However, at 150 mM NaCl Golden Promise maintained significantly higher Mg^{2+} concentrations in young leaf blade ($p<0.05$), old leaf blade ($p<0.01$), higher Mg^{2+} concentrations in stem and root tissues than did Maythorpe. There were no clear differences in individual tissue Mg^{2+} concentrations between Claret and Clansman.

Fig. 2.10 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and whole plant Mg^{2+} concentrations (mg/g dry weight, means ± se). a: Maythorpe (MAY) and Golden Promise (GP); b: Claret (CLT) and Clansman (CLN).

a



b

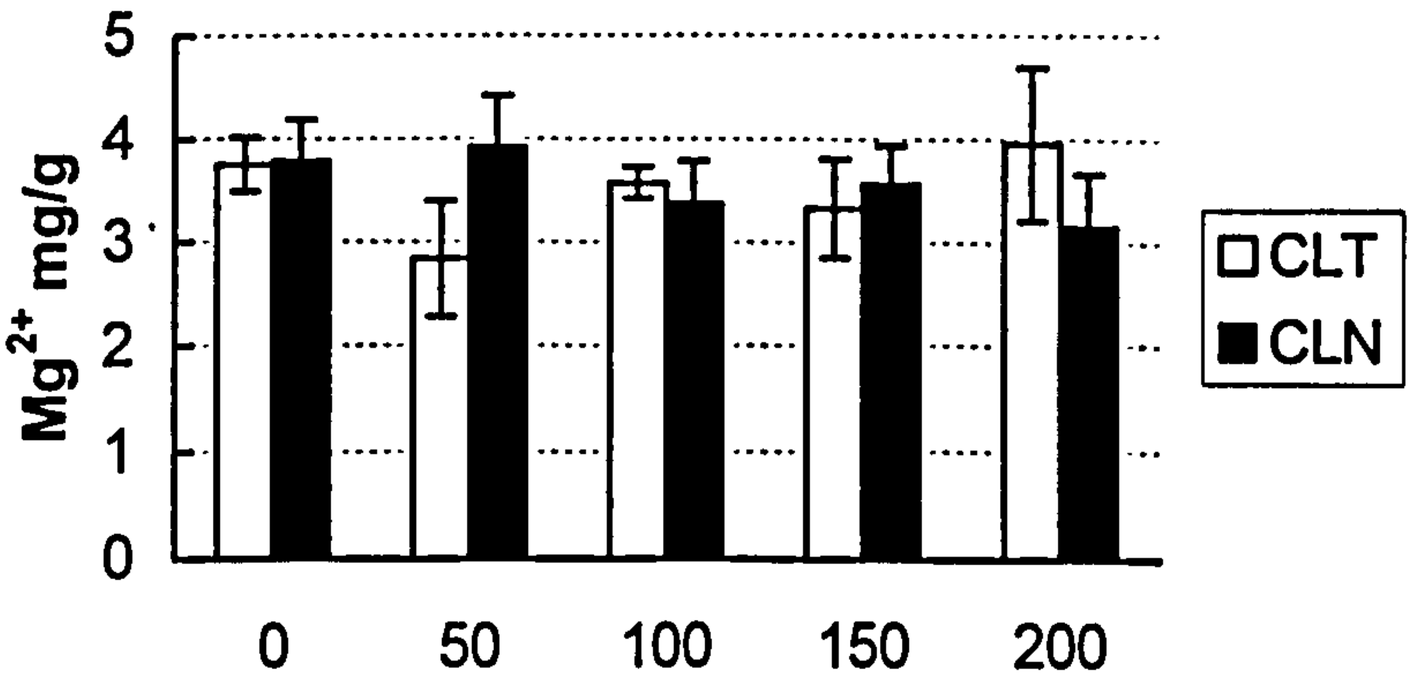


Table 2.13 Mg^{2+} concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	0	1.83±0.02 a	1.98±0.19 a	1.69±0.03 a	1.88±0.19 a
	50	0.99±0.12 b	0.90±0.12 b	0.99±0.05 a	1.74±0.32 a
	100	0.79±0.04 b	0.71±0.12 b	1.09±0.12 a	1.16±0.12 a
	150	0.68±0.08 b	1.63±0.19 ab	1.31±0.40 a	1.21±0.13 a
	200	0.72±0.03 b	1.13±0.25 ab	1.16±0.21 a	1.01±0.08 a
Old leaf blade	0	3.65±0.18 a	3.80±0.43 ab	3.13±0.56 a	3.75±0.66 a
	50	2.15±0.19 b	1.98±0.06 c	2.34±0.23 a	4.15±0.57 a
	100	2.25±0.31 ab	1.50±0.31 c	3.86±0.32 a	2.79±0.63 a
	150	1.57±0.12 b	4.01±0.09 a	2.89±0.45 a	3.39±0.10 a
	200	2.05±0.50 b	2.07±0.59 bc	2.37±0.43 a	3.16±0.27 a
Stem	0	2.80±0.25 a	2.33±0.30 a	3.01±0.71 a	2.60±0.25 a
	50	2.41±0.41 a	1.79±0.21 a	3.04±0.75 a	2.42±0.49 a
	100	2.26±0.63 a	2.34±0.63 a	2.63±0.60 a	2.04±0.22 a
	150	1.96±0.35 a	3.24±0.35 a	3.15±0.69 a	2.72±0.37 a
	200	2.23±0.15 a	1.66±0.48 a	2.27±0.55 a	2.01±0.27 a
Root	0	11.98±2.40 a	6.65±0.43 a	9.03±0.84 ab	15.21±3.72 a
	50	12.03±4.22 a	6.72±2.88 a	5.53±0.74 a	10.89±1.87 a
	100	10.95±5.78 a	7.81±4.00 a	8.73±0.76 ab	10.90±2.33 a
	150	7.03±4.26 a	8.75±2.71 a	8.06±1.91 ab	9.83±1.77 a
	200	11.05±6.21 a	7.29±2.75 a	14.82±3.08 b	10.05±2.25 a

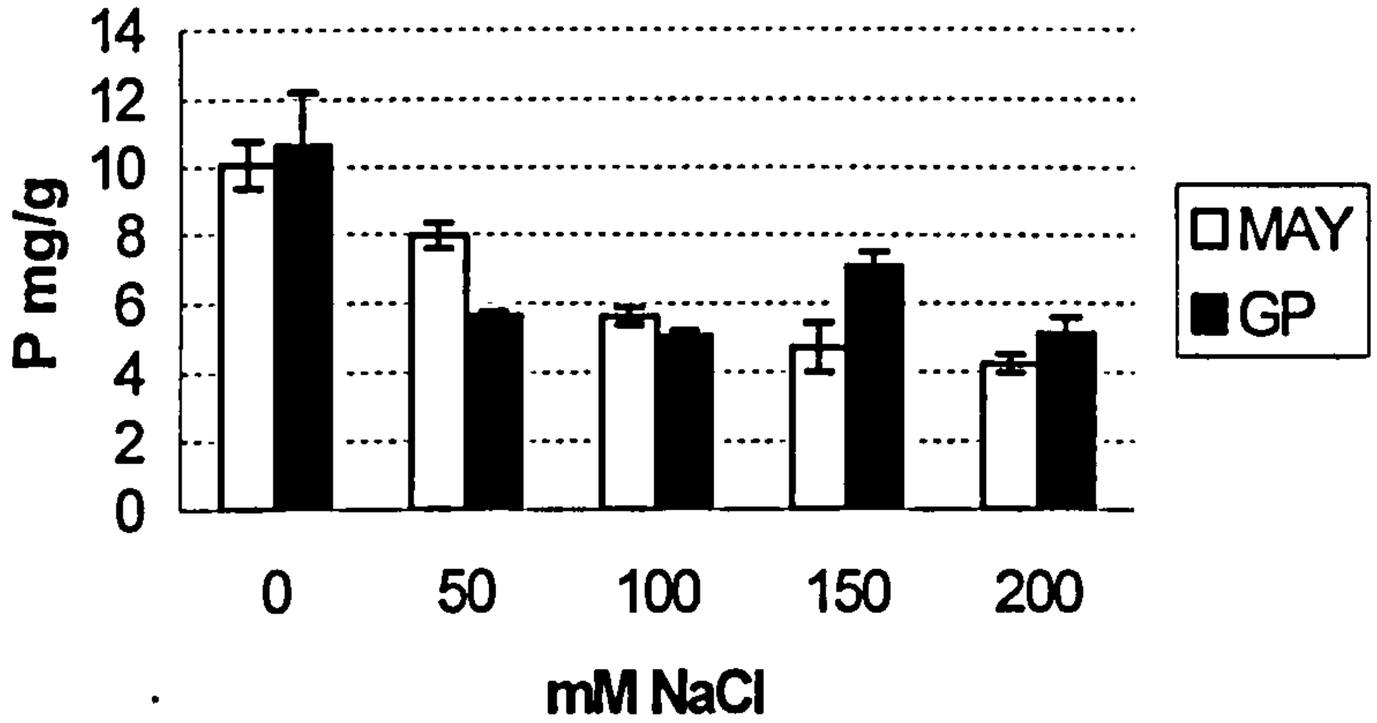
* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.1.7 P concentrations

While whole plant phosphorus concentrations of all cultivars were reduced in response to increasing salinity (Fig. 2.11), there were no significant varietal differences between Maythorpe and Golden Promise and between Claret and Clansman. Similar results were also observed in individual tissues (Table 2.14) although increasing salt caused reductions of P concentrations in individual tissues, especially in root tissues.

Fig. 2.11 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and whole plant P concentrations (mg/g dry weight, means ± se). a: Maythorpe (MAY) and Golden Promise (GP); b: Claret (CLT) and Clansman (CLN).

a



b

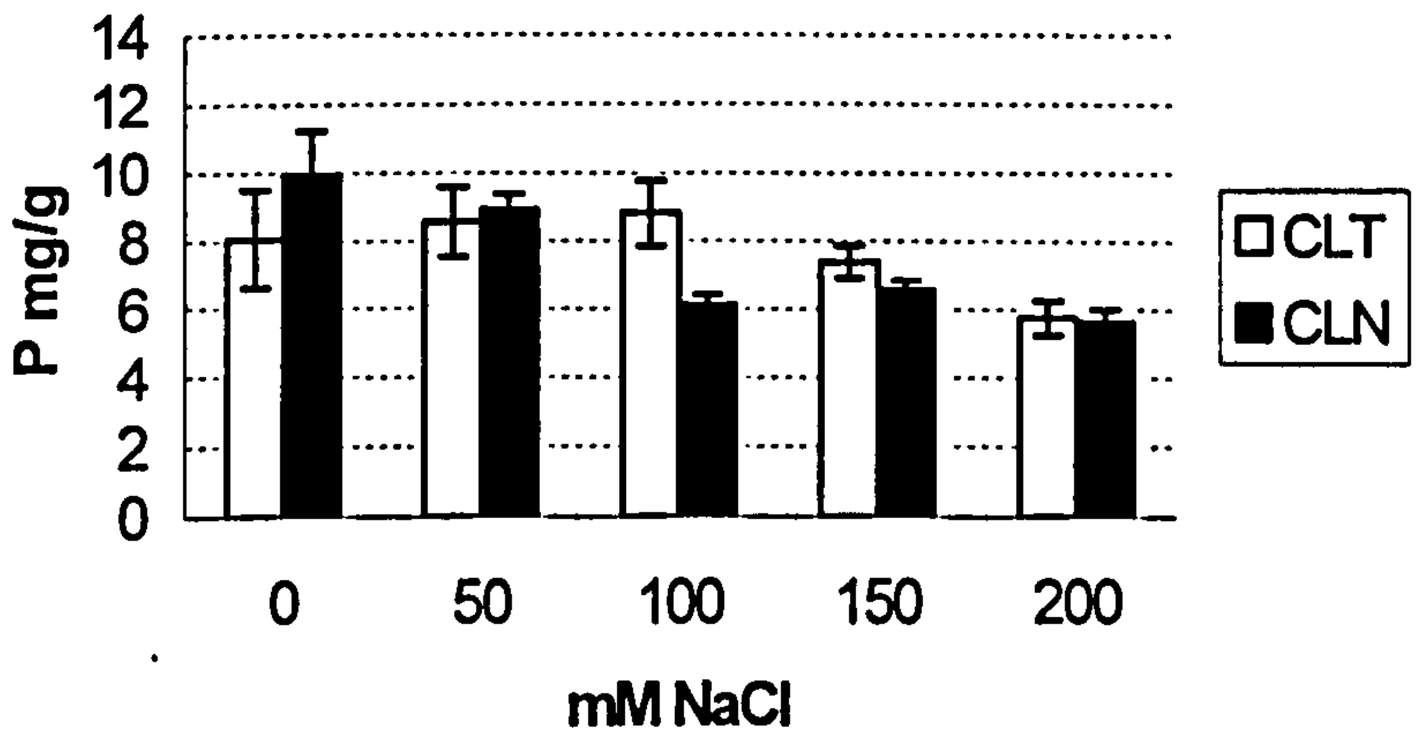


Table 2.14 P concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise	Claret	Clansman
Young leaf blade	0	7.28±0.37 a	8.82±0.82 a	5.75±0.81 a	7.26±0.78 a
	50	6.85±0.71 ab	5.10±0.44 b	7.35±0.76 a	7.88±0.56 a
	100	5.40±0.42 ab	4.74±0.42 b	9.59±1.07 a	6.44±0.46 a
	150	4.71±0.98 ab	6.67±0.36 ab	7.86±0.31 a	6.73±0.29 a
	200	3.45±0.29 b	5.41±0.62 ab	6.53±1.78 a	5.58±0.85 a
Old leaf blade	0	7.34±0.16 a	8.72±1.16 a	5.56±1.09 a	8.60±1.07 a
	50	6.04±0.73 ab	4.06±0.46 a	7.32±1.49 a	8.58±0.63 a
	100	4.56±0.51 ab	4.70±0.42 a	9.46±2.38 a	5.94±0.68 a
	150	4.08±0.93 ab	7.19±1.01 a	7.15±0.67 a	7.41±1.02 a
	200	2.95±0.39 b	4.69±0.61 a	4.08±0.36 a	5.30±0.33 a
Stem	0	8.22±0.31 a	8.69±0.53 a	6.11±0.72 a	8.09±0.38 a
	50	8.12±0.53 a	5.55±0.37 ab	8.17±0.94 a	7.14±0.46 ab
	100	5.86±0.33 ab	5.58±0.37 ab	8.33±0.92 a	5.96±0.29 ab
	150	5.06±0.77 b	7.39±0.43 ab	7.10±0.40 a	5.94±0.19 ab
	200	5.08±0.31 b	5.20±0.31 b	6.03±0.36 a	5.36±0.30 b
Root	0	22.74±1.67 a	22.26±3.50 a	18.22±4.38 a	22.77±2.95 a
	50	11.69±1.79 b	8.89±1.08 b	13.16±0.35 ab	16.34±2.32 ab
	100	5.35±0.65 c	4.77±0.37 b	9.41±0.31 ab	6.67±0.31 c
	150	4.49±0.20 c	6.75±0.37 b	8.45±0.46 ab	7.79±0.38 bc
	200	5.44±0.92 bc	5.36±0.40 b	6.94±0.48 b	7.56±0.24 bc

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2 Experiment two

2.3.2.1 Plant growth

As in Experiment one, the growth of both the relative salt sensitive cultivar Maythorpe and relative salt tolerant cultivar Golden Promise was markedly affected by increasing salinity in experiment two (Table 2.15). The shoot and root dry matter of both cultivars was reduced with increasing salt concentration in the growth medium. A varietal difference was evident that Maythorpe showed a significant reduction in shoot growth at 50 mM NaCl whilst Golden Promise showed a significant reduction at 100 mM NaCl.

Table 2.15 Shoot and root dry weight (g/plant) of barley cultivars Golden Promise and Maythorpe growing 4 weeks under 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

NaCl (mM)	Shoot dry weight		Root dry weight	
	Maythorpe	Golden Promise	Maythorpe	Golden Promise
0	1.33±0.16 a	0.90±0.07 a	0.18±0.03 a	0.13±0.01 a
50	1.06±0.08 b	0.89±0.01 a	0.17±0.02 a	0.13±0.01 a
100	0.77±0.01 .bc	0.59±0.03 b	0.13±0 ab	0.10±0.01 ab
150	0.63±0.02 c	0.44±0.02 bc	0.10±0.01 ab	0.07±0.01 bc
200	0.49±0.05 c	0.37±0.03 c	0.08±0.01 b	0.06±0.01 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

The comparative growth of shoot (CGS) showed clear differences between Golden Promise and Maythorpe in response to increasing salinity (Table 2.16). The CGS of Golden Promise was constantly higher than Maythorpe at all salt treatments but the differences decreased with increasing salt supply to the growth medium. Both cultivars showed similar comparative growth of root (CGR) with increasing salinity. Comparing CGS and CGR, root growth was less affected by the increasing external salt concentrations than shoot in both cultivars. Golden Promise maintained significantly higher YLB:OLB ratios than Maythorpe with increasing salinity. 150 and 200 mM NaCl resulted in a significant decrease in YLB:OLB ratio in Maythorpe, but there was no such effect in Golden Promise (Table 2.17).

Table 2.16 Comparative growth of shoot (CGS) and root (CGR) of Golden Promise and Maythorpe growing 4 weeks under 0, 50, 100, 150 and 200 mM NaCl. Values are means of 3 replicates.

NaCl (mM)	Maythorpe		Golden Promise	
	CGS %	CGR %	CGS %	CGR %
0	100	100	100	100
50	79.72	94.44	99.37	100
100	58.07	72.22	65.44	76.92
150	47.39	55.56	49.37	53.85
200	37.17	44.44	41.41	46.15

Table 2.17 YLB:OLB ratios (dry weight) of Maythorpe and Golden Promise in relation to 0, 50, 100, 150 and 200 mM NaCl.

NaCl (mM)	Maythorpe	Golden Promise
0	1.19±0.06 a	1.30±0.03 a
50	1.06±0.03 a	1.32±0.08 a
100	0.99±0.08 ab	1.03±0.01 a
150	0.63±0.09 b	0.91±0.03 a
200	0.63±0.13 b	0.93±0.23 a

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.2 Chlorophyll concentration

Golden Promise contained higher chlorophyll concentrations (in the youngest fully expanded leaves) than Maythorpe under both control and saline conditions. These varietal differences were evident across all salt treatments and at both sampling dates (Table 2.18). Increasing salinity did not significantly affect the chlorophyll concentrations of Maythorpe, but 100 mM NaCl resulted a significant increase in chlorophyll concentration of Golden Promise.

Table 2.18 Chlorophyll concentration (Spad reading) of the youngest fully expanded leaves of Golden Promise and Maythorpe under 0, 50, 100, 150 and 200 mM NaCl. Values are means with 9 replicates.

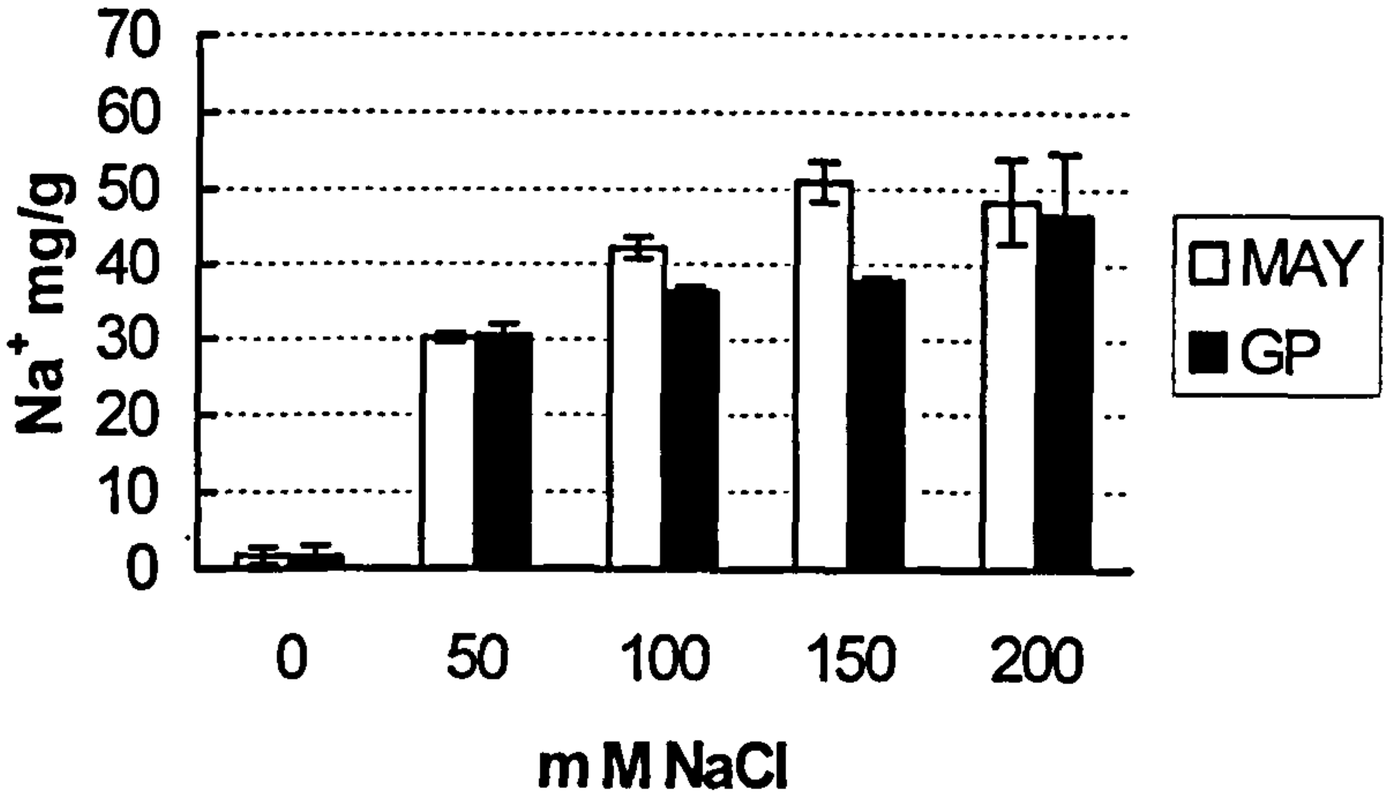
NaCl (mM)	Chlorophyll concentration (Spad reading)			
	Week 2		Week 4	
	Maythorpe	Golden Promise	Maythorpe	Golden Promise
0	40.1±0.8 a	42.0±0.4 a	40.7±0.5 a	44.7±0.7 a
50	39.6±0.8 a	45.0±0.8 a	43.7±1.3 a	46.0±0.8 ab
100	39.2±1.3 a	42.7±0.7 a	49.1±0.6 a	49.3±1.1 bc
150	39.9±1.8 a	41.8±1.1 a	46.3±1.0 a	51.3±1.0 c
200	38.1±1.6 a	41.0±1.8 a	46.3±2.5 a	47.6±1.5 abc

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.3 Na^+ concentrations

Increasing NaCl concentration in the growth media caused a large increase in the whole plant Na^+ concentrations of both cultivars (Fig. 2.12). In the absence of salt both cultivars contained similarly low Na^+, while at a low level of salt (50 mM NaCl) they maintained similar whole plant Na^+ concentrations. The major differences between cultivars occurred between 100 and 150 mM NaCl where Golden Promise contained lower Na^+ than Maythorpe. This difference was reduced at 200 mM NaCl.

Fig. 2.12 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and Na^+ concentrations (mg/g dry weight, means ± se) in whole plants of Maythorpe (MAY) and Golden Promise (GP).



Na^+ concentrations of all individual tissues increased with increasing salinity (Table 2.19). The order of Na^+ concentration among individual tissues under saline conditions was generally young leaf blade < stem < young sheath < root < old leaf blade < old sheath. There was little difference in the Na^+ concentrations of individual tissues in the absence of and presence of a low salt concentration (50 mM NaCl). It was between the concentrations of 100 and 150 mM NaCl that the main varietal differences occurred with Golden Promise exhibiting lower Na^+ concentrations than Maythorpe in all tissues. At 200 mM NaCl both cultivars contained similar Na^+ in individual tissues. No significant differences were observed in the individual tissues between the two cultivars. However, Golden Promise maintained significantly lower Na^+ concentrations in young leaf blade ($p<0.01$), younge sheath ($p<0.01$) and stem ($p<0.05$) tissues than Maythorpe between 0 and 150 mM NaCl treatments.

Table 2.19 Na^+ concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe expressed on dry weight basis in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

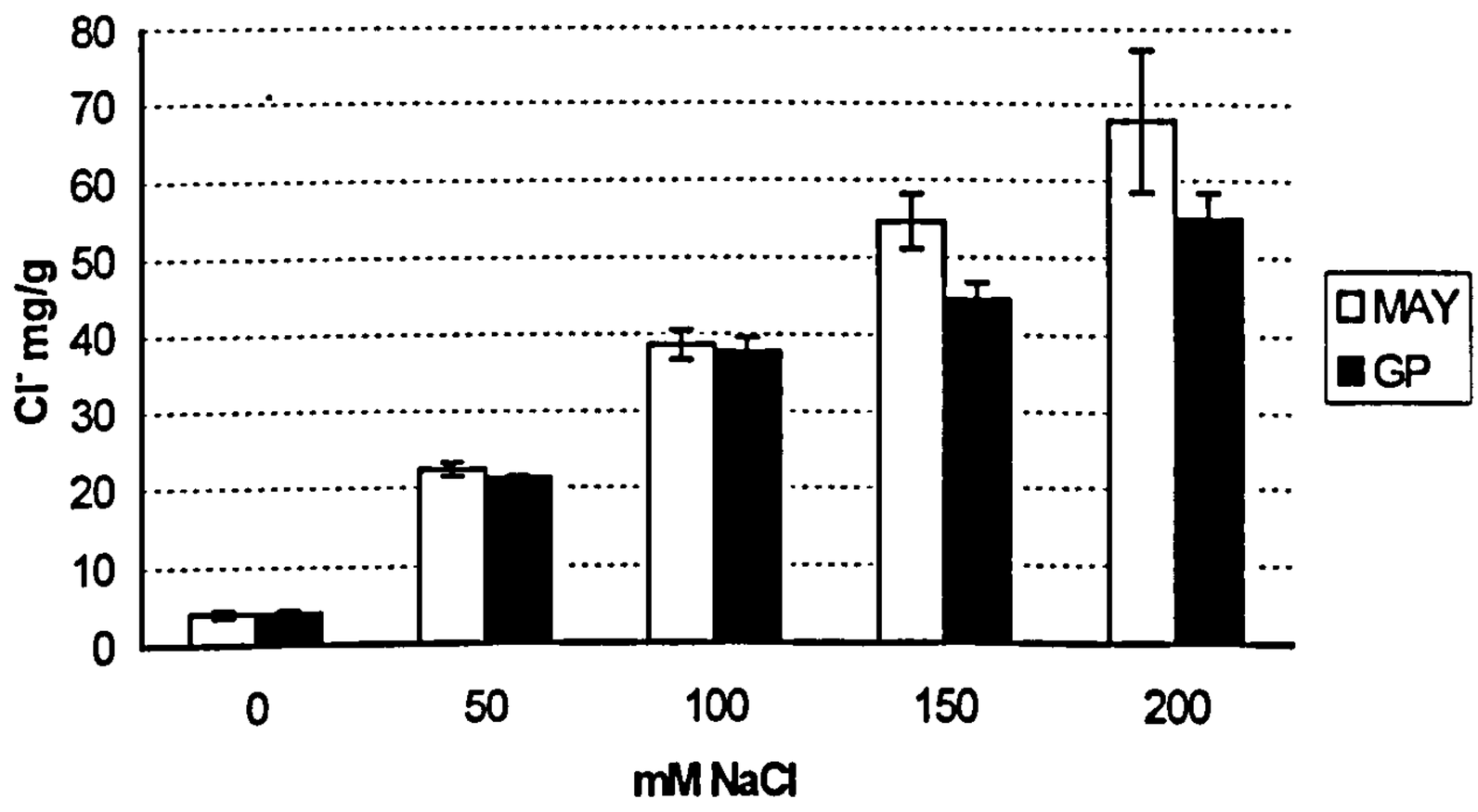
Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	1.34±0.04 a	1.13±0.06 a
	50	20.27±1.69 ab	18.86±1.80 b
	100	25.56±1.96 b	16.02±1.31 b
	150	24.77±3.09 b	16.70±2.18 b
	200	25.98±7.42 b	23.69±5.13 b
Young sheath	0	1.34±0.08 a	1.38±0.06 a
	50	35.50±0.63 b	35.38±1.31 b
	100	44.63±1.17 b	37.18±1.50 b
	150	45.60±5.74 b	30.82±2.06 ab
	200	44.37±7.10 b	50.94±13.01 b
Old leaf blade	0	1.93±0.27 a	1.61±0.02 a
	50	35.25±2.13 b	40.20±0.57 b
	100	51.10±1.32 bc	46.59±2.43 b
	150	58.62±5.49 c	47.61±3.10 b
	200	54.72±7.39 bc	53.48±9.32 b
Old sheath	0	1.75±0.14 a	1.90±0.17 a
	50	46.14±1.98 b	51.94±0.98 b
	100	67.85±1.11 c	63.83±2.27 bc
	150	83.46±7.05 c	66.24±5.38 bc
	200	79.29±3.28 c	76.46±7.99 c
Stem	0	1.65±0.04 a	2.04±0.01 a
	50	29.92±0.81 b	25.87±0.13 b
	100	34.44±1.05 b	31.58±1.53 b
	150	40.46±5.43 b	29.30±2.62 b
	200	42.50±7.64 b	40.67±7.82 b
Root	0	1.97±0.34 a	1.97±0.09 a
	50	28.25±0.37 b	27.3±1.21 b
	100	41.48±1.78 c	41.99±0.56 c
	150	57.22±0.99 d	48.07±2.67 cd
	200	52.29±1.22 d	53.03±1.62 d

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD)

2.3.2.4 Cl^- concentrations

Whole plant Cl^- concentrations of both Golden Promise and Maythorpe were closely related to increasing salinity where higher NaCl concentrations in the growth media caused higher whole plant Cl^- concentrations (Fig. 2.13). No significant difference between the cultivars was observed although Golden Promise maintained lower Cl^- concentrations, especially with increasing salinity.

Fig. 2.13 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and whole plant Cl^- concentrations (mg/g dry weight, means ± se) of Golden Promise (GP) and Maythorpe (MAY).



The Cl^- concentrations in all individual tissues of both cultivars increased in response to increments of NaCl (Table 2.20), this was unlike Na^+ where most of the increase was in response to the first 50 mM of NaCl. The lowest Cl^- concentration was found in root tissue while the highest was in old sheaths. The order of Cl^- was: root < young leaf blade < stem < young sheath < old leaf blade < old sheath. Although no significant differences were observed between cultivars in Cl^- concentrations of individual tissues, at 150 mM external NaCl Golden Promise maintained 14, 9, 17, 11 and 24 % lower Cl^- concentrations than Maythorpe in young leaf blade, young sheath, old leaf blade, stem and root tissues respectively.

Table 2.20 Cl^- concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe expressed on a dry weight basis in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	3.08±0.46 a	3.15±1.16 a
	50	15.78±0.07 ab	15.49±0.29 ab
	100	22.87±0.60 b	18.92±0.79 b
	150	29.57±5.13 bc	25.35±0.84 b
	200	41.68±5.58 c	40.13±5.19 c
Young sheath	0	5.57±0.29 a	8.04±0.87 a
	50	34.30±1.85 b	37.77±1.32 b
	100	51.77±1.67 bc	48.02±0.70 b
	150	62.41±5.92 cd	56.99±2.52 b
	200	75.65±7.11 d	89.64±12.62 c
Old leaf blade	0	4.95±0.78 a	3.65±0.80 a
	50	27.20±1.47 a	26.66±1.09 a
	100	57.27±2.95 b	59.01±3.83 b
	150	76.38±3.72 bc	63.59±3.01 b
	200	90.33±11.35 c	83.62±10.17 b
Old sheath	0	6.29±0.95 a	7.59±0.90 a
	50	38.89±1.78 b	38.98±0.81 b
	100	66.51±3.40 c	76.89±5.90 c
	150	88.28±2.93 cd	89.43±7.93 c
	200	105.00±8.68 d	102.69±9.00 c
Stem	0	2.66±0.14 a	6.23±0.10 a
	50	19.92±0.81 ab	16.21±0.65 ab
	100	31.41±2.82 bc	29.95±2.64 abc
	150	42.79±2.58 cd	38.18±8.45 bc
	200	55.18±7.08 d	58.57±10.78 c
Root	0	1.18±0.09 a	1.60±0.59 a
	50	9.76±1.20 bc	5.28±0.47 b
	100	5.65±0.37 ab	5.87±0.08 b
	150	12.00±1.47 c	9.14±0.82 c
	200	14.95±1.63 c	15.52±0.87 d

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.5 K^+ concentrations and K^+/Na^+ ratio

Whole plant K^+ concentrations (Fig. 2.14) and K^+ concentrations of individual tissues (Table 2.21) of Golden Promise and Maythorpe were significantly reduced with increasing salinity, there were no significant differences between cultivars either in whole plant or individual tissues although Golden Promise contained lower K^+ than Maythorpe under control conditions. The major reduction occurred between 0 and 50 mM NaCl in all tissues with the exception of young sheath where no significant reduction occurred in both cultivars. After which further increase in NaCl concentrations did not significantly affect the K^+ concentrations of the individual tissues with the exception of root where 100 mM NaCl in Golden Promise and 100 and 150 mM NaCl in Maythorpe resulted in further significant reductions in K^+ concentration.

Fig. 2.14 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and plant K^+ concentrations (mg/g dry weight, means ± se) of Maythorpe (MAY) and Golden Promise (GP).

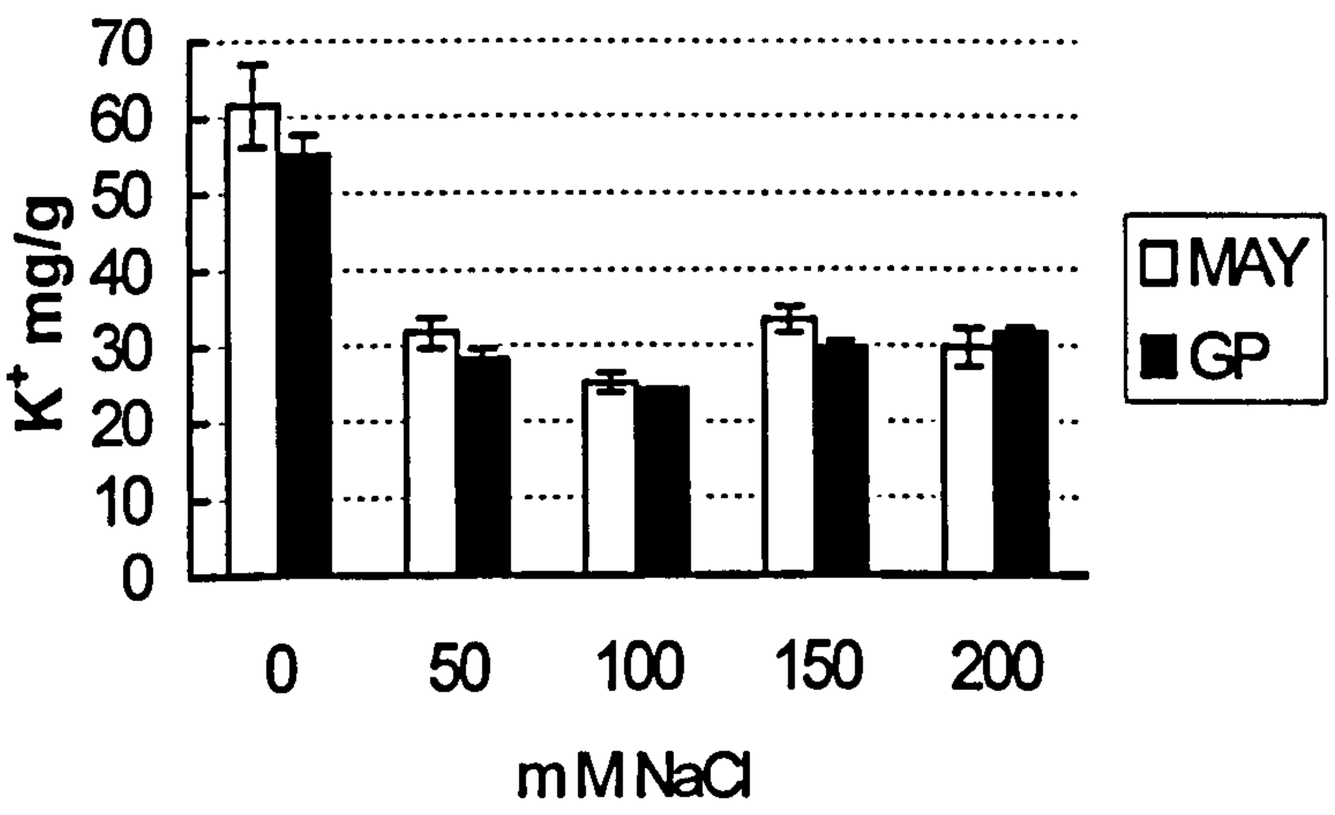


Table 2.21 K^+ concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	60.65±7.58 a	56.94±6.37 a
	50	33.80±3.04 b	31.52±2.70 b
	100	22.02±0.69 b	22.27±1.48 b
	150	32.78±0.60 b	30.80±1.46 b
	200	31.06±2.48 b	32.55±1.08 b
Young sheath	0	65.36±13.57 a	48.69±4.31 a
	50	54.07±3.03 a	46.94±1.94 a
	100	36.12±1.17 a	40.46±2.12 a
	150	44.70±1.90 a	50.80±0.77 a
	200	49.74±1.75 a	43.34±4.35 a
Old leaf blade	0	62.73±4.71 a	59.57±5.60 a
	50	26.33±2.68 b	20.21±0.45 c
	100	29.81±3.40 b	26.70±1.04 bc
	150	41.90±4.24 ab	33.42±1.37 bc
	200	35.71±6.49 b	40.00±1.68 b
Old sheath	0	69.00±13.96 a	47.99±2.23 a
	50	32.31±0.48 b	28.87±1.84 b
	100	22.95±1.79 b	21.23±1.51 b
	150	30.06±0.80 b	25.07±1.55 b
	200	23.13±1.78 b	23.62±1.55 b
Stem	0	62.25±4.46 a	57.52±2.36 a
	50	31.92±0.91 b	26.75±0.65 b
	100	25.36±1.84 b	26.83±0.74 b
	150	28.96±1.35 b	31.44±0.63 b
	200	32.27±3.08 b	32.01±2.60 b
Root	0	52.75±0.92 a	49.55±4.08 a
	50	26.20±0.55 b	25.08±0.75 b
	100	15.75±1.50 c	14.95±0.07 c
	150	9.82±0.95 d	10.30±1.25 c
	200	8.08±0.21 d	8.49±0.63 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

In the presence of salt Golden Promise maintained significantly higher (K^+/Na^+) ratios than Maythorpe only in young leaf blade ($p<0.05$) (Table 2.22). The major differences occurred at the treatments of 100 and 150 mM NaCl. There were no significant differences between cultivars in the K^+/Na^+ ratios of other tissues.

Table 2.22 K^+/Na^+ ratios of individual tissues of Golden Promise and Maythorpe growing 4 weeks under 50, 100, 150 and 200 mM NaCl. Values are means of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	50	1.67 a	1.68 a
	100	0.87 b	1.41 a
	150	1.37 ab	1.89 a
	200	1.33 ab	1.52 a
Young sheath	50	1.52 a	1.33 a
	100	0.81 b	1.09 a
	150	1.02 ab	1.66 a
	200	1.18 ab	1.02 a
Old leaf blade	50	0.74 a	0.50 a
	100	0.58 a	0.57 ab
	150	0.71 a	0.71 ab
	200	0.65 a	0.78 b
Old sheath	50	0.70 a	0.56 a
	100	0.34 b	0.33 b
	150	0.37 b	0.39 ab
	200	0.29 b	0.31 b
Stem	50	1.07 a	1.03 a
	100	0.73 a	0.85 a
	150	0.74 a	1.09 a
	200	0.76 a	0.79 a
Root	50	0.93 a	0.92 a
	100	0.38 b	0.36 b
	150	0.17 c	0.21 c
	200	0.15 c	0.16 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.6 Ca^{2+} concentrations and Ca^{2+}/Na^{+} ratios

Salinity induced a reduction in whole plant Ca^{2+} concentration in both Golden Promise and Maythorpe (Fig. 2.15). This reduction occurred between 0 and 50 mM NaCl with little change as salinity was increased further. There was no significant difference in whole plant Ca^{2+} concentrations between Golden Promise and Maythorpe. Golden Promise maintained significantly higher Ca^{2+} ($p<0.05$) in both young and old sheath than the corresponding tissues of Maythorpe (Table 2.23). No significant difference between cultivars was observed in young leaf blade, old leaf blade, stem and root tissues. Under saline conditions Golden Promise maintained higher Ca^{2+}/Na^{+} ratios in young leaf blade ($p<0.05$), young sheath ($p<0.01$) old leaf blade ($p<0.01$), old sheath ($p<0.01$) and root ($p<0.05$) than Maythorpe with differences being greatest at high salinity (Table 2.24).

Fig. 2.15 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and whole plant Ca^{2+} concentrations (mg/g dry weight, means ± se) of Maythorpe (MAY) and Golden Promise (GP).

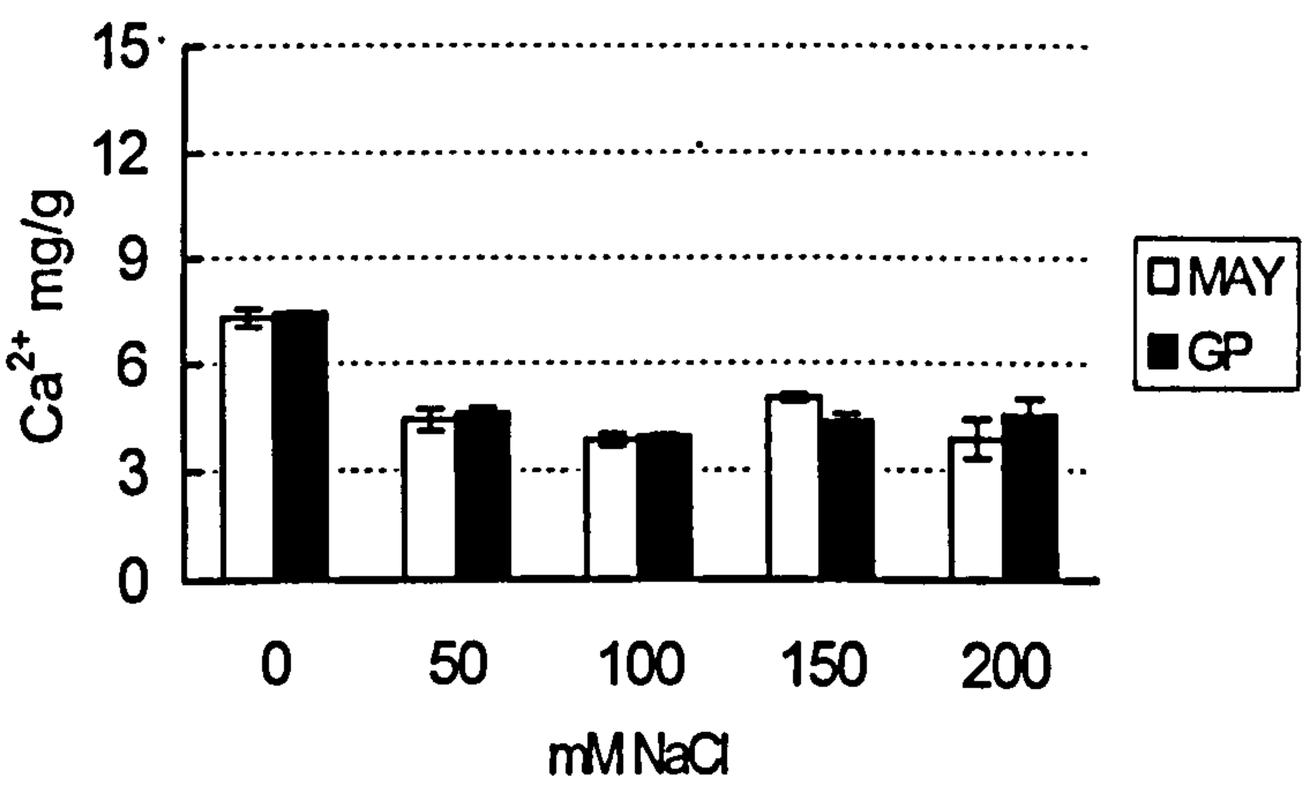


Table 2.23 Ca^{2+} concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	4.53±0.06 a	4.52±0.09 a
	50	2.19±0.10 b	2.29±0.26 bc
	100	1.88±0.10 b	1.47±0.04 c
	150	2.52±0.29 b	2.21±0.26 bc
	200	2.64±0.47 b	2.64±0.27 b
Young sheath	0	5.34±0.24 a	5.33±0.70 a
	50	5.01±0.24 ab	5.37±0.25 a
	100	3.77±0.13 b	3.99±0.05 a
	150	3.95±0.16 ab	4.99±0.31 a
	200	3.89±0.48 b	4.72±0.44 a
Old leaf blade	0	13.03±0.31 a	13.28±0.37 a
	50	6.66±0.65 b	7.53±0.19 b
	100	6.48±0.47 b	7.00±0.11 b
	150	7.71±0.22 b	6.99±0.41 b
	200	5.38±1.19 b	6.91±0.48 b
Old sheath	0	5.25±0.36 a	5.83±0.28 a
	50	4.02±0.16 a	4.27±0.22 a
	100	4.08±0.43 a	4.43±0.30 a
	150	4.81±0.12 a	5.07±0.12 a
	200	3.90±0.42 a	5.18±0.53 a
Stem	0	2.15±0.09 ab	2.43±0.10 a
	50	2.07±0.13 ab	2.07±0.04 a
	100	1.73±0.05 b	1.85±0.15 a
	150	2.36±0.10 a	1.83±0.07 a
	200	1.88±0.16 ab	2.26±0.28 a
Root	0	6.41±0.52 a	6.63±0.18 a
	50	5.34±0.17 a	5.61±0.40 a
	100	3.05±0.02 b	3.56±0.10 b
	150	3.71±0.38 b	3.63±0.11 b
	200	3.04±0.18 b	3.38±0.13 b

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

Table 2.24 Ca^{2+}/Na^{+} ratios in individual tissues of Golden Promise and Maythorpe growing 4 weeks under 50, 100, 150 and 200 mM NaCl. Values are means of 3 replicates.

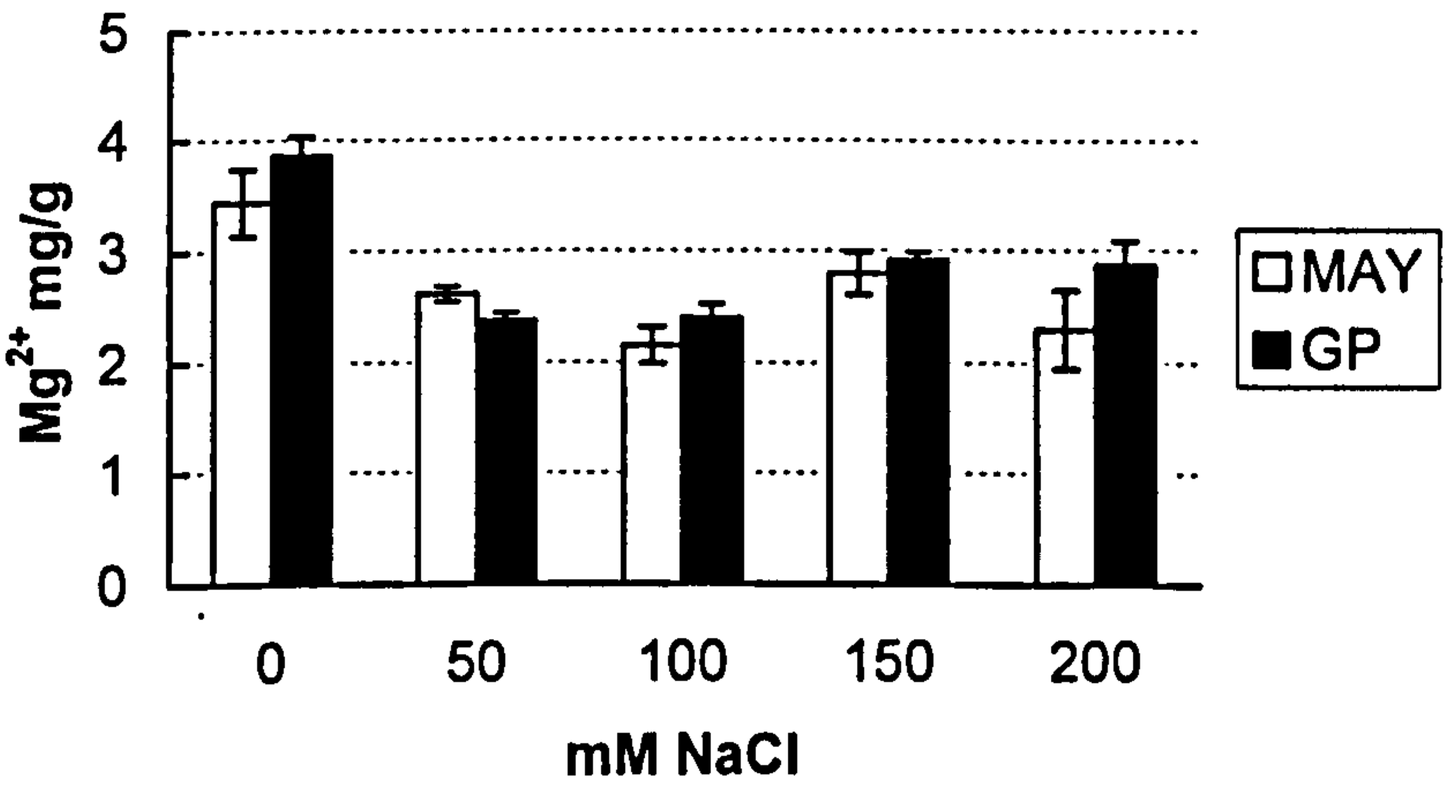
Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	50	0.11 a	0.12 a
	100	0.07 a	0.09 a
	150	0.10 a	0.13 a
	200	0.11 a	0.12 a
Young sheath	50	0.14 a	0.15 a
	100	0.08 b	0.11 a
	150	0.09 ab	0.16 a
	200	0.09 ab	0.10 a
Old leaf blade	50	0.19 a	0.19 a
	100	0.13 b	0.15 ab
	150	0.13 b	0.15 ab
	200	0.10 b	0.13 b
Old sheath	50	0.09 a	0.08 a
	100	0.06 ab	0.07 a
	150	0.06 b	0.08 a
	200	0.05 b	0.07 a
Stem	50	0.07 a	0.08 a
	100	0.05 a	0.06 a
	150	0.06 a	0.06 a
	200	0.05 a	0.06 a
Root	50	0.19 a	0.21 a
	100	0.07 b	0.08 b
	150	0.06 b	0.08 b
	200	0.06 b	0.06 b

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.7 Mg^{2+} concentrations

Whole plant Mg^{2+} concentration of both cultivars decreased as NaCl concentration was increased from 0 to 50 mM with little change thereafter (Fig. 2.16). Although there was no significant difference in Mg^{2+} concentration between the two cultivars, Golden Promise maintained higher Mg^{2+} concentrations than Maythorpe with the exception of 50 mM NaCl.

Fig. 2.16 Relationship between salt treatments of 0, 50, 100, 150 and 200 mM NaCl and plant Mg^{2+} concentrations (mg/g dry weight, means ± se) of Maythorpe (MAY) and Golden Promise (GP).



The effects of salinity on Mg^{2+} concentrations were varied and depended on the type of tissues and the salt concentrations (Table 2.25). Increasing salinity resulted in a reduction in Mg^{2+} concentration in young leaf blade, old leaf blade and root tissues, where the major decrease occurred between 0 and 50 mM NaCl. However, increasing external salinity caused an increase in Mg^{2+} concentration in old sheath and stem tissues of both cultivars. Golden Promise contained significantly higher Mg^{2+} than Maythorpe in young sheath ($p<0.01$) and old leaf blade ($p<0.05$) tissues.

Table 2.25 Mg^{2+} concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	2.12±0.07 a	2.20±0.08 a
	50	1.40±0.11 b	1.62±0.14 bc
	100	1.20±0.05 b	1.16±0.04 c
	150	1.72±0.10 ab	1.65±0.09 bc
	200	1.57±0.20 ab	1.68±0.11 b
Young sheath	0	1.90±0.10 a	2.04±0.32 a
	50	2.08±0.03 a	2.15±0.05 a
	100	1.82±0.08 a	1.90±0.02 a
	150	2.28±0.02 a	2.61±0.04 a
	200	2.20±0.26 a	2.82±0.32 a
Old leaf blade	0	4.12±0.16 a	4.97±0.14 a
	50	2.41±0.26 ab	2.86±0.06 b
	100	2.66±0.25 ab	3.20±0.02 b
	150	3.13±0.23 ab	3.55±0.18 b
	200	2.29±0.65 b	3.35±0.23 b
Old sheath	0	1.96±0.12 a	1.88±0.20 a
	50	1.58±0.07 a	2.01±0.24 ab
	100	2.83±0.35 ab	3.41±0.43 bc
	150	4.80±0.57 c	4.70±0.19 c
	200	3.97±0.37 bc	4.66±0.33 c
Stem	0	2.24±0.23 a	2.07±0.20 a
	50	2.31±0.04 a	2.96±0.20 b
	100	2.59±0.31 a	2.24±0.12 ab
	150	2.87±0.20 a	2.58±0.10 ab
	200	2.27±0.12 a	2.63±0.29 ab
Root	0	8.36±2.33 a	9.61±1.29 a
	50	7.20±0.34 ab	3.70±0.90 b
	100	2.63±0.52 b	3.01±0.70 b
	150	2.64±0.34 b	3.26±0.20 b
	200	2.49±0.51 b	3.06±0.27 b

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.2.8 P concentrations

Increasing salinity had little effect on the P concentration in whole plant (Fig. 2.17) or individual tissues (Table 2.26). Golden Promise maintained significantly higher ($p<0.01$) P concentrations in the old leaf blade and old sheath tissues, but significantly lower ($p<0.01$) P concentration in the stem tissue than in the corresponding tissues of Maythorpe with increasing salinity. No significant differences in P concentrations were observed between the two cultivars in the young leaf blade, young sheath and root tissues.

Fig. 2.17 Relationship of salt treatments of 0, 50, 100, 150 and 200 mM NaCl and plant P concentrations (mg/g dry weight, means ± se) of Maythorpe (MAY) and Golden Promise (GP).

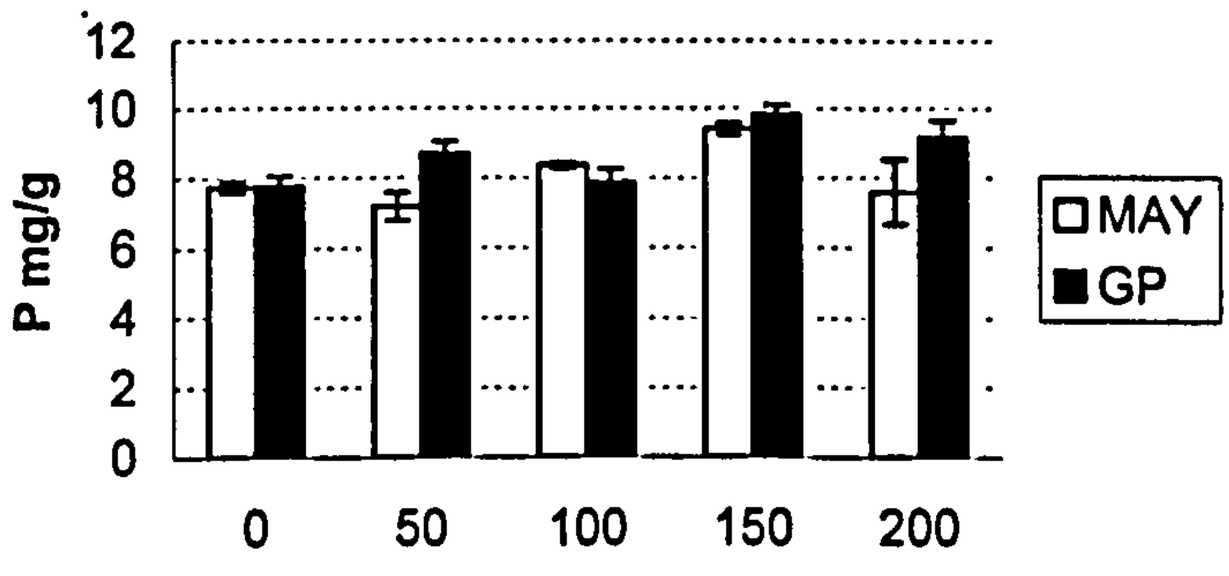


Table 2.26 P concentrations (mg/g dry weight) in individual tissues of Golden Promise and Maythorpe in response to 0, 50, 100, 150 and 200 mM NaCl. Values are means (±se) of 3 replicates.

Tissue	NaCl (mM)	Maythorpe	Golden Promise
Young leaf blade	0	7.76±0.29 a	7.60±0.54 a
	50	7.19±0.45 a	8.63±0.80 a
	100	7.30±0.45 a	6.10±0.30 a
	150	9.25±0.31 a	8.85±0.58 a
	200	8.43±0.51 a	8.97±0.65 a
Young sheath	0	6.91±0.32 a	6.38±0.70 a
	50	7.33±0.35 ab	7.55±0.56 a
	100	7.00±0.15 a	7.18±0.02 a
	150	8.61±0.36 b	7.55±0.32 a
	200	7.41±0.18 ab	7.45±0.16 a
Old leaf blade	0	8.73±0.22 a	9.21±0.50 a
	50	7.63±0.61 a	10.23±0.24 ab
	100	11.40±0.38 a	11.15±0.77 ab
	150	10.47±0.58 a	13.55±0.67 b
	200	7.67±2.03 a	10.41±1.17 ab
Old sheath	0	5.35±0.22 a	5.74±0.03 a
	50	4.68±0.14 a	5.98±0.11 a
	100	5.73±0.15 a	5.89±0.31 a
	150	7.32±0.18 b	7.57±0.41 b
	200	5.96±0.51 ab	7.42±0.32 b
Stem	0	7.68±0.21 a	6.89±0.12 a
	50	8.49±0.43 a	7.80±0.33 a
	100	8.07±0.38 a	7.00±0.40 a
	150	8.00±0.12 a	7.85±0.30 a
	200	7.25±0.42 a	8.15±0.38 a
Root	0	8.35±0.15 ab	7.86±0.14 ab
	50	7.56±0.22 ab	8.49±0.27 b
	100	6.91±0.17 a	7.15±0.29 a
	150	9.27±0.77 b	8.20±0.06 abc
	200	8.44±0.58 ab	9.26±0.31 c

* Means within a column followed by letters are significantly different at $p < 0.05$ (LSD).

2.3.3 Experiment three

2.3.3.1 Na^+ distribution in leaf cells in response to salinity

Na^+ concentrations of the youngest fully expanded leaves as measured by EDXMA system are presented in Table 2.27. Lower epidermal cells contained lower Na^+ than either upper epidermal or mesophyll cells. The mesophyll cells of Golden Promise contained higher Na^+ than the corresponding cells of Maythorpe, which was an unexpected result. However because the dispersive energy of Na^+ is very low, and the lack of sensitivity for the EDXMA system at low energy levels, the results were very dependent on the leaf surface section. Therefore, the data in Table 2.27 can not reliably be used to explain Na^+ distributions in leaf cells.

Table 2.27 Na^+ distribution between different cells within the youngest fully expanded leaf of Golden Promise and Maythorpe. Values expressed as P/B and are means (±se) of 5 replicates.

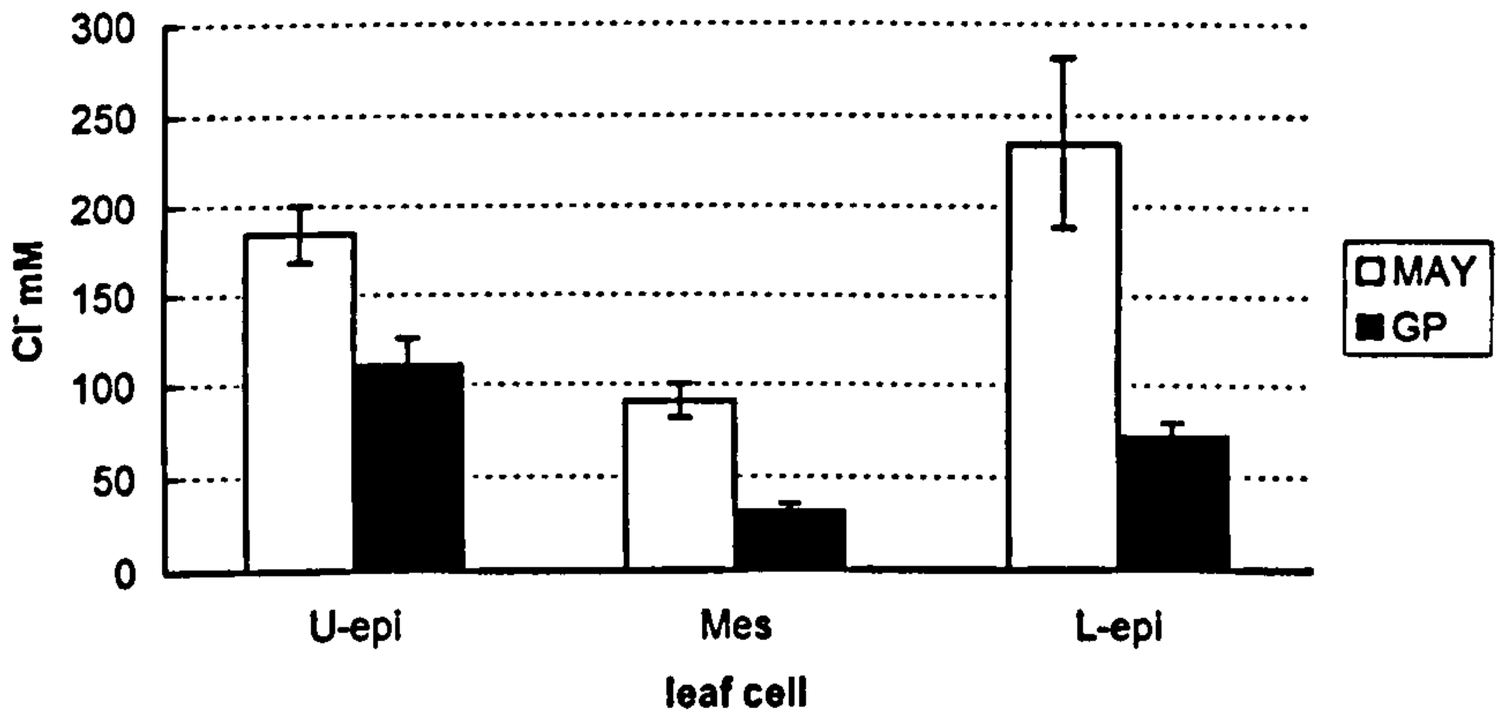
	Maythorpe (P/B)	Golden Promise (P/B)
Upper epidermal cell	1.85 ± 0.14	1.64 ± 0.41
Mesophyll cell	1.33 ± 0.09	1.66 ± 0.12
Lower epidermal cell	1.09 ± 0.12	1.09 ± 0.10

*P/B means peak (height):background (height)

2.3.3.2 Cl^- distribution in leaf cells in response to salinity

Cl^- cellular distributions within the youngest fully expanded leaf of both Golden Promise and Maythorpe grown at 150 mM NaCl as measured by EDXMA are presented in Fig. 2.18. Generally, both upper and lower epidermal cells contained higher Cl^- than mesophyll cells in both Golden Promise and Maythorpe. The relative salt tolerant cultivar Golden Promise contained significantly lower Cl^- in its upper epidermal cells ($p<0.05$), lower epidermal cells ($p<0.01$) and mesophyll cells ($p<0.05$) than the corresponding cells of the relatively salt sensitive cultivar Maythorpe.

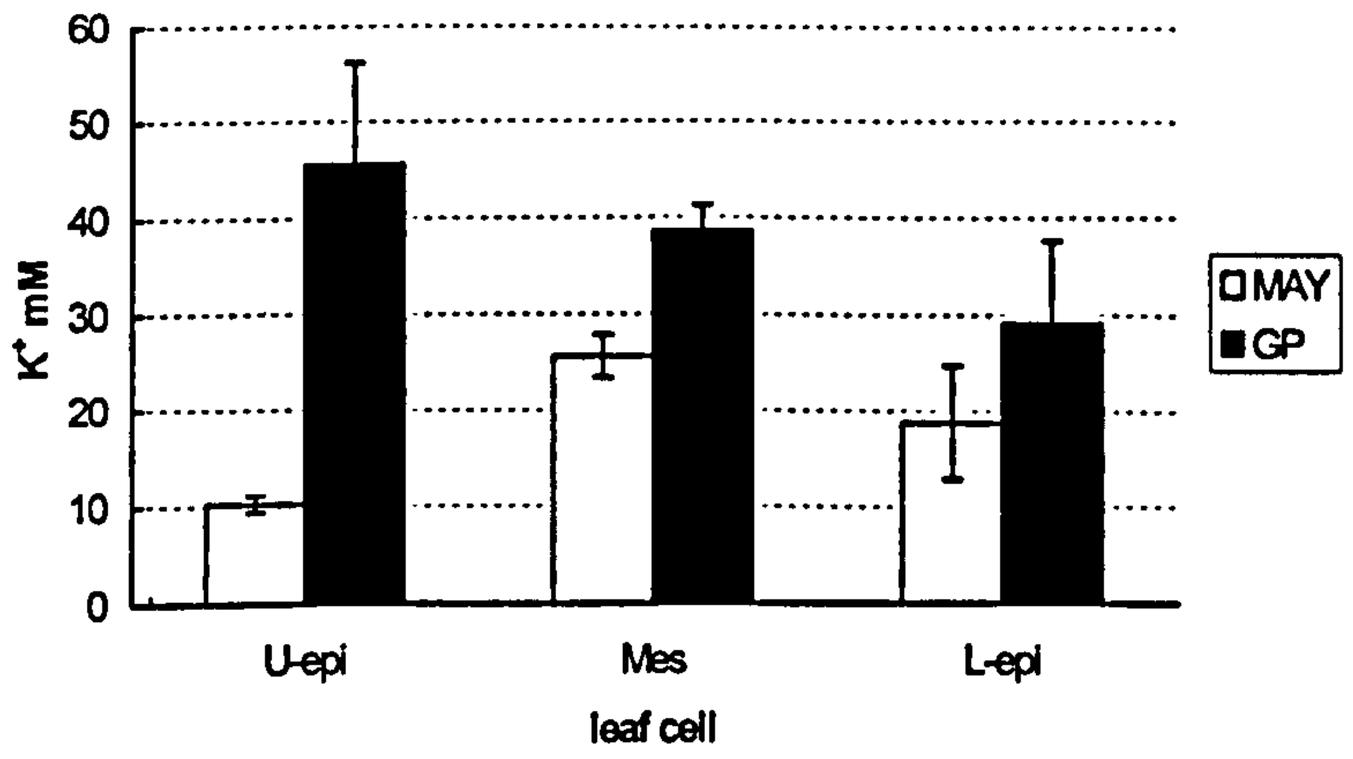
Fig. 2.18 Chloride distribution between different cells within the first fully expanded leaf of Golden Promise (GP) and Maythorpe (MAY). U-epi, Mes and L-epi represent upper epidermal, mesophyll and lower epidermal cells respectively. Data are means (±se) of 5 replicates.



2.3.3.3 K^+ distribution in leaf cells responded to salinity

K^+ cellular concentrations in the youngest fully expanded leaves of Golden Promise and Maythorpe under 150 mM are shown in Fig. 2.19. Golden Promise maintained significantly higher K^+ concentrations in upper epidermal ($p<0.01$), mesophyll ($p<0.01$) and lower epidermal ($p<0.05$) cells than the corresponding cells of Maythorpe.

Fig. 2.19 K^+ distribution between different cells within the youngst fully expanded leaf of Golden Promise (GP) and Maythorpe (MAY). U-epi, Mes and L-epi represent upper epidermal, mesophyll and lower epidermal cells respectively. Data are means (±se) of 5 replicates.



2.4 Discussion

The two isogenic barley cultivars Golden Promise and Maythorpe showed different growth characteristics under control conditions. Golden Promise was semi-dwarf with erect leaves, while Maythorpe was tall with long drooping leaves. These growth differences were presumably a result of the *GPert* mutation, which was also related to salt tolerance (Pakniyat *et al.*, 1997a). In this study, increasing NaCl concentrations in the growth medium resulted in significant reductions in the growth of Golden Promise, Maythorpe, Clansman and Claret, but there were large differences between cultivars. Golden Promise (relatively salt tolerant) maintained higher comparative growth of shoot (CGS) than Maythorpe (relatively salt sensitive) under high salinity (between 100 and 200 mM NaCl) in both experiments. Because higher CGS means smaller differences between control and treatments in dry weight, the above results indicated that increasing salinity had less effect on the growth of Golden Promise compared to that of Maythorpe. Clansman (relatively salt tolerant) and Claret (relatively salt sensitive) also showed similar results with the former maintaining higher CGS than the latter cultivar in the presence of salt. This high CGS in Golden Promise was also reflected in higher YLB:OLB ratios and chlorophyll concentrations than in Maythorpe. Golden Promise showed slightly higher YLB:OLB ratios in response to NaCl concentrations of 50 and 100 mM NaCl in experiment one and higher YLB:OLB ratios at all salt treatments in experiment two than Maythorpe. This would indicate that Golden Promise possessed capability to maintain more actively growing young leaves under salt stress than Maythorpe. Furthermore, Golden Promise also maintained significantly higher chlorophyll concentrations in the youngest fully expanded leaf than Maythorpe with increasing salinity. Therefore, Golden Promise could potentially produce more carbohydrates with more actively growing leaves and higher chlorophyll

concentrations. As a consequence, salt tolerance in Golden Promise was enhanced compared to Maythorpe. Similar to shoot growth, the comparative growth of root (CGR) of both cultivars was also affected by increasing salinity. The root growth in Golden Promise was less affected than the shoot growth, but in Maythorpe the results from experiment one showed that both shoot and root growth were similarly affected by increasing salinity and the results from experiment two showed that root growth was less affected than shoot. From the observation in this study and those by Forster *et al.* (1994) and Pakniyat *et al.* (1997a), it appears that 200 mM NaCl is too high a concentration to be able to identify differences in salt tolerance in the cultivars used in this study, so the following discussion will mainly focus on the treatments of 50 to 150 mM NaCl, where the greatest varietal differences in response to salinity were observed.

Sodium concentration has been shown to be an important indicator of differences in salt tolerance of plants in response to salinity (Greenway and Munns, 1980; Gorham *et al.*, 1985; Neumann, 1997). Pakniyat *et al.* (1997a) demonstrated that barley *GPert* mutants contained significantly less Na^+ in their shoots than non-*GPert* cultivars at 175 mM NaCl. A major aim of the present study was to further investigate this and locate specific tissue differences. When plants were separated into four tissues in experiment one, Golden Promise maintained significantly lower Na^+ concentrations than Maythorpe in young leaf blade, old leaf blade, stem and root tissues. It is crucial for salt tolerant plants to restrict Na^+ flux into meristematic and actively growing and photosynthesising cells (Hasegawa *et al.*, 2000), because high Na^+ accumulation in the tissues of glycophyte plants could cause serious damage as a result of osmotic stress, ion imbalance and ionic toxicity. Salt tolerant plants possess capabilities to exclude

Na^+ ions from actively growing leaves by partitioning extra Na^+ ions into old tissues and vacuoles (Yeo and Flowers, 1986; Yeo *et al.*, 1990; Amtmann and Sanders, 1999). Golden Promise maintained significantly lower Na^+ concentrations, especially in the young leaves, with increasing salinity when compared to Maythorpe. This would enable leaves to perform comparatively normal functions such as photosynthesis with increasing salinity.

Golden Promise is a semi-dwarf cultivar with smaller stature than its tall parent Maythorpe with large leaf area (Forster *et al.*, 1994). Therefore differences in transpiration may account for the differences in Na^+ uptake as it has been shown that Na^+ absorption by roots was affected mainly by the transpiration rate (Marschner, 1995). Besides transpiration, there could be other mechanisms to manipulate Na^+ uptake in barley because it was observed that raising NaCl concentrations in the growth medium beyond 100 mM did not cause further increase of Na^+ concentrations in young leaf blades in all cultivars. Previous studies with barley have suggested that salt tolerance in this plant was related to the ability to selectively partition Na^+ into old leaves and sheaths and K^+ into growing tissues (Greenway, 1962; Stassart and Bogemans, 1987). The results in experiment one were in agreement with these observations and showed that old leaf blades and stem contained much higher Na^+ concentrations than young leaf blades. Because in experiment one the stem fraction included a mixture of sheath and stem, to further understand the differences in Na^+ partitioning in barley, sheath was separated and treated as an individual tissue in experiment two. From experiment two, Golden Promise showed significantly lower Na^+ concentrations in young and actively growing tissues, *i.e.* young leaf blade, young sheath and stem than Maythorpe between 0 and 150 mM NaCl treatments. No

significant varietal differences in Na^+ concentrations were observed in old leaf blades and old sheaths in which most of the Na^+ ions were selectively partitioned. Old leaf blades and old sheaths contained high Na^+ concentrations without significant varietal differences, but young leaf blades and young sheaths contained less Na^+ with significant varietal differences. The results suggested that Golden Promise possessed the ability to minimise excess Na^+ ions entering its young tissues compared to Maythorpe. This restriction could occur between the young stem and old stem or between the joint of young sheath and stem. Rausch *et al.* (1996) suggested that Na^+ is compartmentalised in vacuoles until their loading capacity is reached after which cytosolic concentrations start to increase in response to increasing NaCl supply. Thus significantly lower Na^+ concentrations in the young tissues of Golden Promise implied that this cultivar could maintain low cytosolic Na^+ concentrations and enhance salt tolerance. Additionally, the possibility of selective redistribution of Na^+ in phloem as suggested by Munns *et al.* (1986) and Wolf and Jeschke (1986) could also contribute to different Na^+ partitioning between Golden Promise and Maythorpe. Similar results were observed between Clansman and Claret where the former cultivar maintained significantly lower Na^+ concentrations in whole plant and individual tissues than the latter in response to increasing salt concentrations in growth medium. So the present work would suggest that it is the maintenance of low Na^+ concentrations in young actively growing tissues that could be an important mechanism in the enhanced salt tolerance of Golden Promise compared to Maythorpe.

Besides Na^+, Cl^- accumulation in plant tissues could also be toxic to growth and development. Both experiments one and two showed Cl^- concentrations were much different to Na^+ in that increasing Cl^- concentrations were observed with increments of

NaCl up to 200 mM NaCl while the largest increases in Na^+ concentrations were observed at low external NaCl treatments. No significant differences in Cl^- concentrations were observed in individual tissues between Golden Promise and Maythorpe. The former cultivar maintained lower Cl^- concentrations in most of the tissues than Maythorpe at 150 mM external NaCl in experiment two with the exception of old sheaths. Preferential accumulation of Cl^- in sheath tissue relative to blade tissue was found in wheat, maize, sorghum and barley (Boursier *et al.*, 1984; 1987). In young leaf blade tissue Golden Promise contained 14 % lower Cl^- than Maythorpe, but no such differences between these cultivars were observed in experiment one. Maythorpe maintained higher Cl^- concentrations than Golden Promise probably because of the difference in leaf area between the two cultivars. Further investigation of Cl^- concentrations in the cells of the youngest fully expanded leaf showed that Golden Promise contained significantly lower Cl^- in both epidermal and mesophyll cells than Maythorpe at 150 mM external NaCl. Huang and Van Steveninck (1989) reported that the salt tolerant barley cultivar California Mariout showed higher ability to exclude Cl^- from mesophyll cells than the salt sensitive cultivar, Clipper and Cl^- preferentially accumulated in vacuoles of epidermal cells to maintain a low level in the mesophyll cells. Under high salinity, epidermal cells accumulated more Cl^- than mesophyll cells in barley (Fricke *et al.*, 1996). Leigh and Storey (1993) suggested that reduction in growth at high salinity could be related in part to the accumulation of Cl^- in mesophyll cells disrupting Cl^- sensitive metabolic processes. The maintenance of low Cl^- in mesophyll cells in the youngest fully expanded leaf of Golden Promise could contribute to the enhanced salt tolerance of this cultivar, even though no significant varietal differences in Cl^- concentration of whole plant or individual tissues was observed.

Potassium is an important nutrient in the maintenance of cell turgor and enzyme activation (Smart *et al.*, 1996; Maathuis *et al.*, 1997; Thiel and Wolf, 1997; Liang, 1999). Under a saline environment it is suggested that Na^+ competes with K^+ in membrane transport processes (Schachtman and Liu, 1999) and replacement of K^+ by Na^+ from enzymes results in a loss of activity. Salinity was found to induce potassium deficiency in spinach and maize (Chow *et al.*, 1990; Botella *et al.*, 1997). In the present study, increasing salt concentrations in the growth medium resulted in reductions of K^+ concentrations in whole plants and individual tissues of both Golden Promise and Maythorpe, although no significant varietal differences were observed. This is in agreement with Pakniyat *et al.* (1997) who found no differences between Golden Promise and Maythorpe for shoot K^+, Ca^{2+}, Mg^{2+} and P at either 0 or 175 mM NaCl. The results implied that K^+ concentrations under salt stress may not play an important role in differential salt tolerance between the cultivars. Clansman and Claret showed similar results without significant varietal differences in K^+ concentrations in whole plant and tissue levels in response to increasing salinity. At the cellular level, however, Golden Promise contained significantly higher K^+ in mesophyll cells and both upper and lower epidermal cells of the youngest fully expanded leaf than the corresponding cells of Maythorpe at 150 mM NaCl. These significant cellular differences in this specific young actively growing leaf could be important for the cells to perform relatively normal functions under salt stress leading to enhanced salt tolerance in Golden Promise.

K^+/Na^+ ratio has been reported to be an important feature in the salt tolerance of barley, wheat and other plants (Rathert *et al.*, 1981; Gorham *et al.*, 1990a; Gorham, 1990b; Niu *et al.*, 1995). Salt tolerant plants are generally associated with selective

uptake of potassium into the shoot (Davis, 1984; Schachtman *et al.*, 1989; Gorham *et al.*, 1990b) and are able to maintain higher K^+/Na^+ ratios (Flowers *et al.*, 1986; Gorham, 1990a; Ayala *et al.*, 1997). In experiment one, Golden Promise maintained significantly higher K^+/Na^+ ratios in all tissues, which indicated that Golden Promise possessed the capability to maintain better K^+ and Na^+ balance under high salinity compared to Maythorpe. It was further confirmed by experiment two where Golden Promise showed significantly higher K^+/Na^+ ratios than Maythorpe in young actively growing tissues *e.g.* young leaf blade, young sheath, and stem tissues. Thus maintaining a favourable K^+/Na^+ ratio in the young tissues of Golden Promise might play an important role in salt tolerance compared to Maythorpe. Similar results were also observed between Clansman and Claret where the former maintained significantly higher K^+/Na^+ ratios than Claret in young leaf blade, old leaf blade and stem tissues.

Calcium is important in the maintenance of membrane integrity and hence K^+/Na^+ discrimination (Epstein, 1998). Golden Promise maintained significantly higher Ca^{2+}/Na^+ ratios than Maythorpe in most of the tissues under salt stress, especially in young tissues, although no significant varietal differences were observed between Golden Promise and Maythorpe. Higher Ca^{2+}/Na^+ ratios could allow Golden Promise to maintain normal membrane functions, which could limit Na^+ entering cells and increase the partitioning of Na^+ into the vacuoles. So, like K^+/Na^+ ratios, Ca^{2+}/Na^+ ratios could also be an important factor contributing to differential salt tolerance. Similarly, Clansman maintained higher Ca^{2+}/Na^+ ratios than Claret in all tissues in response to salt stress. Ca^{2+} plays an important role in membrane stabilisation and signalling (Bressan and Hasegawa, 1998). Although the role of Ca^{2+} in NaCl stress adaptation is not well defined, externally supplied Ca^{2+} reduces the toxic effects of

NaCl (Zhong and Läuchli, 1994). Under high salinity, Na^+ was shown to displace Ca^{2+} from the plasma membrane of cotton root hairs (Cramer *et al.*, 1985) and Ca^{2+} inhibits inward-rectifying K^+ channels, that may reduce the Na^+ influx (Schroeder *et al.*, 1994). Therefore high Ca^{2+}/Na^+ ratios could mean that less Ca^{2+} ions, potentially, would be displaced by Na^+ from plasma membrane, which could in turn limit Na^+ uptake.

It is clear that ionic balance plays an important role in salt tolerance in plants as evidenced from high salt induced potassium deficiency in maize (Botella *et al.*, 1997) and calcium enhanced plant salt tolerance in *A. thaliana* (Liu and Zhu, 1998). The maintenance of and the restriction of Na^+ to young actively growing tissues, the selective partitioning of Cl^- and K^+ within leaf tissues and the maintenance of high K^+/Na^+ and Ca^{2+}/Na^+ ratios in young tissues are all mechanisms, which could contribute to the enhanced salt tolerance of Golden Promise and Clansman. Pakniyat *et al.* (1997a) demonstrated that the *ari-e*.GP mutation acted as a major gene for salt tolerance in a large collection of different barley cultivars. Hence a single gene that influenced growth habit also had a major effect upon a stress tolerance phenotype. The inference of the present work is that alteration in Na^+ and Cl^- partitioning, K^+/Na^+, and Ca^{2+}/Na^+ ratios in Golden Promise could be controlled by the *ari-e*.GP mutation, while Na^+ exclusion is the key trait of salt tolerance.

Chapter 3

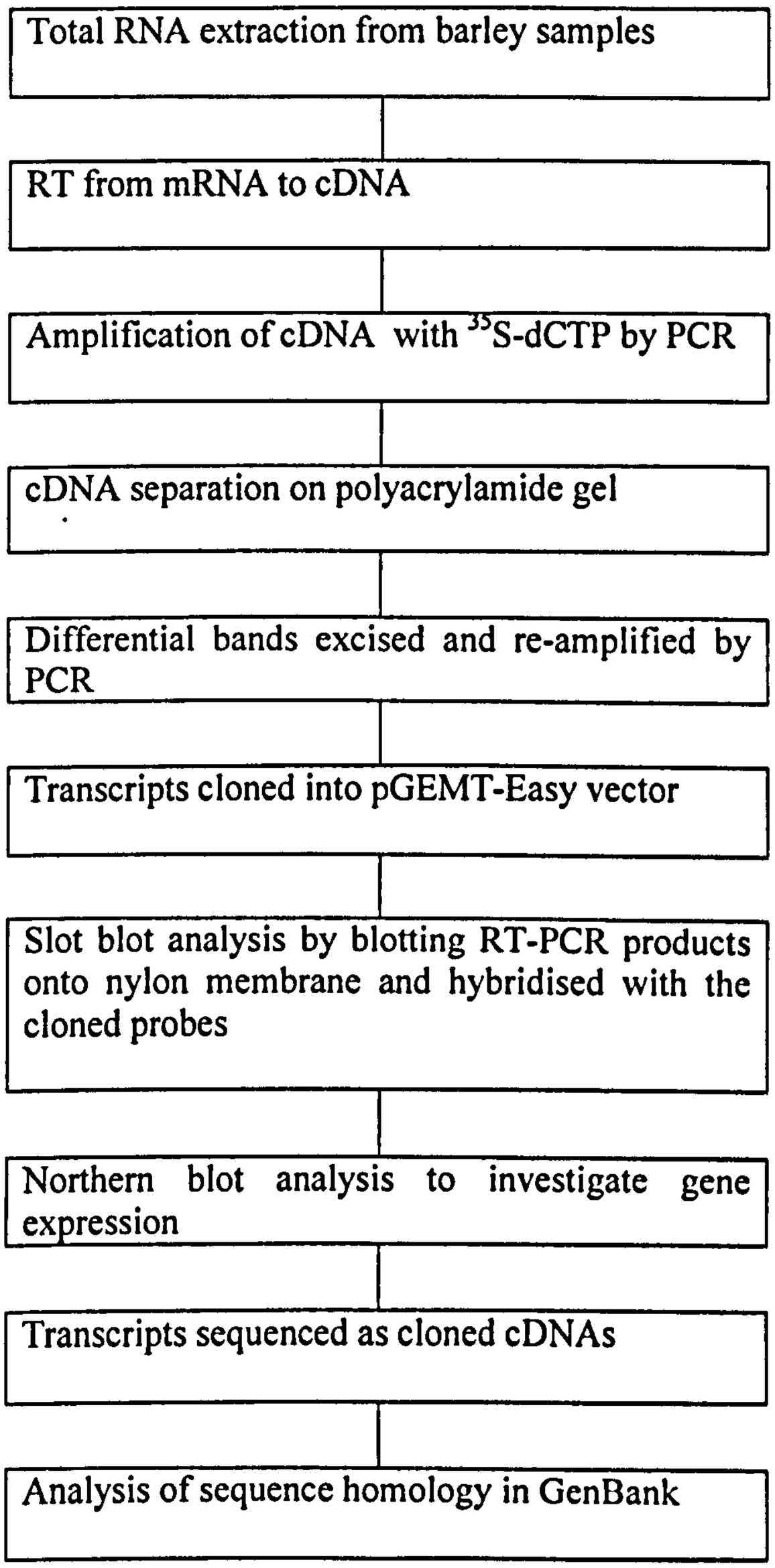
Investigation of differentially expressed genes

3.1 Introduction

A number of physiological and morphological differences between the two near isogenic barley cultivars caused by the *GPert* gene mutation have been determined (Forster *et al.*, 1994; Pakniyat *et al.*, 1997a). To understand the molecular mechanisms of salt tolerance, comparative studies of mRNA and protein levels are key to exploring the nature of the differences. In order to identify changes in gene expression between Golden Promise and Maythorpe in relation to salt stress, mRNA differential display, which was invented by Liang and Pardee (1992), is an ideal technique. The method relies upon the direct visualisation on polyacrylamide gels of reverse transcribed, radiolabelled cDNA products of different mRNA populations produced in the two cultivars and/or under varied growth conditions. Because of the advantage that multiple samples can be analysed simultaneously (Von der Kammer *et al.*, 1999) and the fact that both up- and down-regulated genes can be identified in the same experiment, it has become a favoured approach in the identification of differentially expressed genes in plants (Petrucco *et al.*, 1996; Truesdell and Dickman, 1997; Shen *et al.*, 2001). However, many laboratories have realized the various limitations of differential display, which include poor reproducibility of patterns on polyacrylamide gels (Debouck, 1995), co-migration of bands, and large numbers of false positive signals (Debouck, 1995; DeFrancesco, 1998). Some significant improvements have been made to minimise the number of false positives and facilitate the cloning of polymerase chain reaction (PCR) products (Bauer *et al.*, 1993; Linskens *et al.*, 1995;

Miele *et al.*, 1998), several adjustments and refinements of each individual step in the differential display have been described since its original publication (Liang *et al.*, 1993; Vietor and Huber 1997). In this study a modified mRNA differential display technique was applied and the major procedures are shown in Fig. 3.1.

Fig. 3.1 A summary of the key steps in differential display.



In this study some modifications to the method have been used. In RT and PCR one-base and two-base anchored oligo-dT primers were compared for choosing suitable oligo-dT primer. Twelve Primer combinations of oligo-dT and decamer random primers were applied to select efficient ones for further analysis in order to identify differential bands effectively. Normal PCR of RT products with non-radioactive nucleotides was carried out to check PCR efficiency before incorporating the radioactive ^{35}S-dCTP, this was done to optimise conditions for PCR prior to using radioactivity. To minimise false signals, treble-check was performed. Autoradiography of polyacrylamide gels confirmed that target bands were cut correctly. Fragments were cloned into pGEM-T Easy vector to develop probes and Southern (slot) blot analysis was performed to confirm positive differential fragments. Finally, Northern blot analysis was used to investigate gene expression pattern of the differential transcripts.

Microarrays can also be used to investigate gene expression in plants. This method was recently developed and depends on the production of a collection of partial or complete clones. Suitable probes, for example, produced from cDNA molecues of mRNA populations from particular growth conditions can then be used to identify key inducible genes. This powerful method can give simultaneous information on the expression of many hundreds of genes (Brown and Botstein 1999; Kehoe *et al.* 1999; Lipshutz *et al.* 1999; Harmer *et al.*, 2000). With decreasing costs, arrays for an increasing number of species are becoming available, microarray technology is another important technique in understanding gene expression in plants. Microarrays for barley were not commercially available at the time the present work was carried out. The objectives of this part of the project were to isolate, clone and characterise

cDNA sequences that were differentially expressed between Golden Promise and Maythorpe in response to salinity.

3.2 Materials and Methods

3.2.1 Plant material

Seeds of Golden Promise and Maythorpe were germinated as in section 2.2.1.1 The treatments used are shown in Table 3.1.

Table 3.1 Treatments for molecular analysis. For long-term salt stress, the first increment of 25 mM NaCl was added after 5 days growing in full strength Hoagland and Arnon nutrient solution. Boron and mannitol were used to mimic physiological effects of salinity in terms of ion toxicity and osmotic stress. GP means Golden Promise and MAY means Maythorpe.

	Treatment	Initial
Control	0 mM NaCl for as long as LTSS treatment	GP-CK
		MAY-CK
Long-term salt stress	150 mM NaCl for four weeks after final concentrations of 150 mM NaCl were reached (NaCl added in daily increments of 25 mM).	GP-LTSS
		MAY-LTSS
Short-term salt stress	150 mM NaCl for one day, plants were grown in control nutrient solution until one day before harvest when 150 mM NaCl was added to the nutrient solution.	GP-STSS
		MAY-STSS
Boron toxicity	15 mg l^{-1} added to control nutrient solution for 2 weeks prior to harvest.	GP-BT
		MAY-BT
Osmotic stress	0.1 M mannitol added to control nutrient solution for 2 weeks prior to harvest.	GP-MS
		MAY-MS
Recovery	150 mM NaCl for four weeks (as LTSS) and then 0 mM NaCl for two weeks	GP-Rec
		MAY-Rec

Each treatment had 12 plants in three trays, all trays were randomly arranged. Plant growing conditions were as 2.2.1.3. At harvest, sterilised gloves were worn, plants were separated into young leaves and roots with sterilised scissors, packed in foil paper and immediately frozen in liquid nitrogen. Plant samples were stored in a -70 °C freezer until use. All treatments were harvested at the same time with the exception of the recovery treatment where harvest was two weeks later.

3.2.2 RNA extraction

All equipment to be used for RNA extraction and its subsequent analysis was soaked in 0.1 % diethyl pyrocarbonate (DEPC) water for at least 24 hours followed by double autoclaving to prevent the action of RNases. All equipment and solutions were stored in a separate clean place. The bench was also sterilised by spraying with 70 % ethanol. At all times during the work disposable gloves were worn. General molecular biology techniques were followed according to Sambrook *et al.* (1989).

Total RNA from barley fully expanded young leaves and roots and garlic shoot tissues was prepared using an Ambion Totally RNA kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Garlic was used as a control as this had previously been used to successfully isolate a gene transcript from garlic tissue with mRNA differential display (Wei *et al.*, 2002a). About 0.7 g fresh tissue sample was ground to a fine powder in liquid nitrogen with a DEPC-treated mortar and pestle, then 1 ml of denaturation solution (supplied with kit) was added and ground until the sample was defrosted. 0.7 ml of the resulting slurry was transferred to a 1.5 ml fresh RNase-free Eppendorf. 0.7 ml phenol : chloroform : isoamyl alcohol (IAA) (25:24:1, pH 6.6) solution was added and the mixture was shaken vigorously for 1 min (min)

prior to being stored on ice for 5 min. The mixture was then centrifuged at 12,000 ×g for 5 min at 4 °C. The upper aqueous phase was transferred to a new RNase-free sterile Eppendorf after which 70 µl sodium acetate solution (3 M, pH 4.5) was added and mixed well by inverting tubes several times. Then 0.7 ml of acid phenol : chloroform (5:1, pH 4.7) was added and the mixture was vigorously shaken for 1 min, stored on ice and centrifuged at 12,000 ×g for 5 min at 4°C. The upper aqueous phase was transferred to a fresh RNase-free 1.5 ml Eppendorf and an equal volume of isopropanol was added. The solution was mixed well and maintained at -20 °C for 60 min. The solution was then centrifuged at 12,000 ×g for 15 min at 4 °C and the supernatant was carefully removed by pipette. Residual salts were removed by adding 0.3 ml 70 % ethanol and gently flicking the tube for about 1 to 3 min. The RNA was recovered by centrifugation for 10 min at 12,000 ×g, the ethanol was carefully and thoroughly removed and the pellet was dried at room temperature. The pellet was re-suspended in 70 µl RNase-free water with heating at 70 °C with intermittent vortexing. Contaminating DNA was removed by adding 10 units of RNase-free DNase (Promega, Madison, WI, USA) and incubating for 20 min at 37 °C following heating at 75 °C for 10 min with stop solution (Appendix 20) to stop DNase activity. The yield and purity of RNA was determined by measuring absorbance at 260 and 280 nm. RNA extracts were split into aliquots and stored at -70 °C. Subsamples of the RNA were visualised by electrophoresis on 1 % agarose gels.

3.2.3 Reverse transcription

RT was carried out with MMLV reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's instructions. One-base anchored oligo-dT primer and two-base anchored oligo-dT primer were used in this study (Table 3.2). Total RNA

(0.4 μg) and 1 μl of a poly-T primer (equivalent to 20 pmole) were added to a 0.5 ml RNase-free microtube. The volume was made up to 21 μl using RNase-free water. Primers were annealed to mRNA by heating the mixture at 65 °C for 5 min followed by cooling on ice immediately. 5 μl of 5× MMLV reaction buffer (Appendix 21), 0.5 μl (100 units) MMLV reverse transcriptase, 2 μl of dNTPs solution (containing 1 mM of dATP, dCTP, dGTP and dTTP) and RNase-free water were added to the mixture to a final volume of 50 μl. The tube was mixed by vortexing and incubated at 42 °C for 1 hr and stored at -20 °C.

Table 3.2 One base and two-base anchored oligo-dT primers used in mRNA RT and the following PCR. ·

Anchored Primer	Sequence
PT1	5'-$T_{(11)}$A-3'
PT2	5'-$T_{(11)}$G-3'
PT3	5'-$T_{(11)}$C-3'
PT4	5'-$T_{(12)}$CG-3'
PT5	5'-$T_{(12)}$GA-3'
PT6	5'-$T_{(12)}$AG-3'

3.2.4 Initial PCR to test conditions

3.2.4.1 Amplification of *Aspergillus nidulans agaA* DNA from pAGA

A positive control reaction was carried out to test PCR conditions such as *Taq* DNA polymerase, reaction buffer and dNTPs. In this reaction, pUC19 containing a genomic clone of the *Aspergillus nidulans* arginase gene (*agaA*) (Borsuk *et al.*, 1999) was used as template; primers (forward primer 5'CAG TCG TCA CCG TCA AGA3' and reverse primer 5'TGA AAT CGC TGACTGACG3'), PCR components (Table 3.3) and reaction protocol (Table 3.4) were utilised to amplify *agaA* DNA. The resulting PCR products were run on a 1 % agarose gel containing 0.05 μg/ml of ethidium bromide in 0.5×TBE buffer (Appendix 16), the gel was then visualised on a UV transilluminator.

Table 3.3 Components of PCR reaction mixture to amplify the *agaA* DNA from *A. nidulans*.

Reagent	**Volume added (μl)**
$MgCl_2$ (25 mM)	5
10×DNA Polymerase buffer ($MgCl_2$ free)	5
dNTP mixture solution (5 mM)	2
Forward primer	1
Reverse primer	1
pAGA	7.7 (0.1 μg DNA)
Taq polymerase (1.25 unit / 2 μl) (Promega)	2
dH_2O	Up to 50

Table 3.4 PCR programs for amplification of *agaA* DNA from *A. nidulans*.

Program	Temperature (°C)	Time (min)	Cycle
1*	95	5	1
2	95	0.5	40
	53	2	
	72	2	
3	72	10	1

* Hot start step without *Taq* DNA polymerase before adding *Taq* and running program 2.

3.2.4.2 Preparation of positive control DNA

Escherichia coli cells (*DH5α*) were transferred to a Luria-Bertani (LB) medium plate, which was prepared by pouring about 30 ml of autoclaved LB medium (Appendix 6) plus 15 g agar/1000 ml. The plate was incubated overnight at 37 °C. One colony was transferred to 5 ml LB medium and the solution was incubated overnight at 37 °C in a shaker at 250 ×g. 200 µl of the cultured solution was added into a 0.5 ml microtube and centrifuged at 12,000 ×g for 5 min. The pellet was washed with 200 µl of dH_2O and centrifuged. The supernatant was discarded and 100 µl dH_2O was added to resuspend the pellet prior to boiling for 10 min. The DNA solution was stored at -20 °C until required.

3.2.4.3 Initial PCR to test poly-T and random primers

The cDNA samples produced by RT with poly-T anchored primers were amplified using the following PCR protocols with different poly-T anchored primer (Table 3.2) / random primer (Table 3.5) combinations. 20 µl RT product was used as template DNA. The components of the PCR mixture are listed in Table 3.6 and PCR programs

are listed in Table 3.7. Afterwards, 10 µl of PCR product was loaded on a 1 % agarose gel, the rest of the PCR product was stored at -20 °C.

Table 3.5 Sequences of random primers used in PCR.

Random Primers	Sequence
R1	5'-GTT GCG ATC C-3'
R2	5'-AGC CAG CGA A-3'
R3	5'-TAC AAC GAG G-3'
R4	5'-CTT TCT ACC C-3'
R5	5'-TGG ATT GGT C-3'
R6	5'-GGA ACC AAT C-3'

Table 3.6 Components of a standard PCR reaction for amplification of barley cDNA.

Reagent	Volume added (µl)
$MgCl_2$ (25 mM)	4
10×DNA Polymerase buffer ($MgCl_2$ free)	5
dNTP mixture solution (5 mM)	2
Anchored poly-T primer (20 pmole)	1
Random primer (20 pmole)	1
Template *	20
Taq DNA polymerase (1.25 unit/ 2 µl) (Promega)	2
dH_2O	Up to final volume of 50 µl

* Template produced from RT reaction on 0.4 µg RNA sample

Table 3.7 PCR running programs for amplification of barley cDNA.

Program	Temperature (°C)	Time (min)	Cycle
1*	95	5	1
2	94	1	40
	40	2	
	72	2	
3	72	10	1

* Hot start step without *Taq* DAN polymerase before adding *Taq* and running program 2.

3.2.4.4 Selection of a suitable $MgCl_2$ concentration and comparison of *Taq* DNA polymerases from two companies

$MgCl_2$ concentration is an important factor that affects PCR efficiency. To select a suitable working solution of $MgCl_2$ for PCR of barley samples, three $MgCl_2$ concentrations, 1.5, 2.0, 2.5 mM were tested. The PCR protocol was the same as the above section (3.2.4.3). A comparison of PCR efficiency with two *Taq* DNA polymerases from Promega (Madison, WI, USA) and Flowgen (Lichfield, UK) was carried out by amplifying *E.coli* DNA. The PCR protocol was also the same as the above section (3.2.4.3).

3.2.5 RT-PCR of barley samples with ^{35}S-dCTP

RT was carried out on RNA samples of leaves and roots of all six treatments (CK, STSS, LTSS, Rec, BT and MS) of both Golden Promise and Maythorpe with MMLV

reverse transcriptase (Promega, Madison, WI, USA). Equal amounts of total RNA (0.4 μg) template of different samples was used in the reaction, which was carried out as described in section 3.2.4 using two-base anchored oligo-dT primers of PT4, PT4 and PT6 (Table 3.2). The resulting cDNAs were amplified by PCR with the same anchored oligo-dT primer and a random primer. The primer combinations used in this study were as follows: Pt4/R3, Pt5/R4, Pt6/R5, Pt4/R4. A negative control without DNA template and a positive control with a template of *E. coli* DH5α DNA were used in each PCR. One unit of DyNAzyme^{EXT} DNA polymerase (Flowgen, Lichfild, UK) was used in each PCR reaction with 10 μCi ^{35}S-dCTP (600 Ci/mmole), the PCR components are shown in Table 3.8, and PCR programs were the same as in section 3.2.4.3. The consequent radioactive PCR products were prepared for differential display.

Table 3.8 Components of PCR mixture with ^{35}S-dCTP.

Reagent	Volume added (μl)
10×DNA Polymerase reaction buffer (with 15 mM $MgCl_2$)	5
d(A,G,T)TPs solution (5 mM)	2
^{35}S-dCTP (600 Ci/mmole)	1 (10 μCi)
Anchored poly T primer (20 pmole)	1
Random primer (20 pmole)	1
Template (RT product)	15
DyNAzyme^{EXT} Taq DNA polymerase (1 unit/ 1μl) (Flowgen)	1
dH_2O	24
Mineral oil	50

3.2.6 Separation of cDNAs with polyacrylamide sequencing gels

Polyacrylamide gel stock solution was prepared as in Table 3.9. The stock solution was covered with foil and stored at 4 °C.

Table 3.9 Components of polyacrylamide gel stock solution.

Polyacrylamide gel stock solution	
Acrylamide/bis 40 % (19:1)	150 ml/litre
Urea	480 g/litre
10×TBE buffer (Appendix 1)	100 ml/litre
dH_2O	Make up to 1000 ml

A 6 % polyacrylamide gel (0.4 mm thick and 56 cm long) was prepared following the procedure of Sambrook *et al.* (1989). Two glass plates were carefully cleaned with ethanol and acetone and assembled by placing 2 pieces of 0.4 mm spacer between the glass plates at the edges. Polyacrylamide gel stock solution (60 ml) was filtered through a 0.2 μm sterile filter and 600 μl of fresh 10 % ammonium persulphate solution and 18 μl of N'-tetramethylethylenediamine (TEMED) were added to the solution. A clean syringe was used to homogenise the solution without introducing air bubbles and the solution was left to stand at room temperature for about 1 min. The gel solution was taken up in a syringe carefully and injected with constant pressure into the space between the glass plates. When the space was fully filled without any air bubbles, the gel rig was placed horizontally on a bench and a comb was inserted

backwards at the top of the gel. Both ends of the gel were wrapped in cling film and the gel stored horizontally until set (overnight).

After the comb was carefully removed the gel rig was assembled (Bio Rad) and 575 ml and 350 ml of 1×TBE buffer were placed in the top and the bottom reservoirs separately. The comb was inserted forwards into the top of the gel until the tip of the teeth just penetrated the gel. A syringe was used to wash wells with buffer solution to remove any loose fragments of gel. The gel was pre-run at 1,800 V for 30 min before loading samples. Each ^{35}S-labelled RT-PCR sample (4 µl) was mixed with 1 µl of gel loading buffer (Appendix 2) and heated at 75 °C for 5 min and cooled on ice immediately. After a brief centrifugation the samples were loaded into the wells and the gel was run at 1,800 V for about 6 hr (until the 2nd blue dye had moved near to the bottom of the gel).

After electrophoresis was completed the gel rig was put on a bench and the glass plates were prised apart with the gel normally sticking to one of the plates. Blotting paper was placed onto the gel and gentle pressure applied in order to cause the gel to adhere to the paper. Then the glass plate was removed, the gel was wrapped in cling film and the blotting paper with the gel was dried at 80 °C for 2 hrs on a Bio Rad 583 Gel Dryer. The cling film was removed from the dried gel and the gel was fixed to the negative side of Kodak Biomax MR film by taping and cutting at the top corners in a dark room. The film was exposed at room temperature in a hypercassette for approximately 2 days and developed in 1×Kodak GBX developer solution and fixed in 1×Kodak GBX fixer. The developed film was washed with fresh water for about 15 min and allowed to dry prior to visualisation of bands.

Bands of interest were excised from the gel and eluted (Sambrook *et al.*, 1989). The film and the gel were matched by the cut corners and fixed together by taping. A sharp needle was used to label the bands of interest on the gel through the film. The bands were cut with a clean scalpel. The excised piece of gel along with blotting paper was transferred to a 1.5 ml sterile Eppendorf tube and was incubated in 100 µl dH_2O for 10 min at room temperature. After rehydration of the polyacrylamide gel, the cDNA was diffused out by boiling the gel slice for 15 min. The sample was then centrifuged at 12,000 ×g (4 °C) for 10 min and the supernatant was transferred to a fresh tube. The cDNA was precipitated by adding 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. 5 µl of 10 mg/ml glycogen was used as a carrier. The mixture was then stored at -20 °C for 60 min prior to centrifugation at 12,000 ×g (4 °C) for 15 min. The pellet was dissolved in 10 µl dH_2O and stored at -20 °C.

3.2.7 Cloning differentially expressed cDNA bands

Eluted cDNA bands were amplified by PCR as described in section 3.2.5 with non-labelled nucleotides and the same primer combination as used in the previous amplification. PCR products were visualised on a 1 % agarose gel.

PCR products were purified using a QIAgen quick PCR purification kit (Qiagen Ltd., West Sussex, UK) followed the manufacturer's instructions. Briefly, 5 volumes of buffer PB (Qiagen Ltd., West Sussex, UK) were added to the PCR product and mixed well. The mixture was added to a QIAquick column and centrifuged at 12,000 ×g for 1 min. The flow through was discarded. The column was washed with 750 µl of buffer PE (Qiagen Ltd., West Sussex, UK) and centrifuged at 12,000 ×g for 1 min. The flow through was discarded and the column was recentrifuged for an additional min. The

QIAquick column was placed into a clean 1.5 ml Eppendorf and 50 µl of buffer EB (Qiagen Ltd., West Sussex, UK) (10 mM Tris·Cl, pH8.5) was dispensed directly onto the membrane of the column. The column was left for 1 min at room temperature and then centrifuged at 12,000 ×g for 1 min. The flow through was stored at -20 °C until required for ligation.

Purified PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The ligation reactions were set up as shown in table 3.10. The reactions included 3:1 and 1:3 molar ratios of insert to vector for all isolated cDNA fragments. The reactions were mixed by pipetting and were incubated overnight at 4 °C.

Table 3.10 Ligation reactions for cloning PCR products into pGEM-T Easy Vector.

Component	Standard reaction (µl)	Positive control (µl)	Negative control (µl)
2× ligation buffer	5	5	5
pGEM-T easy (50 ng/µl)	1	1	1
PCR product*	x	0	0
Control DNA insert (4 ng/µl)	0	2	0
T4 DNA ligase (3 Weiss unit/µl)	1	1	1
Sterile distilled water	3 - x	2	2
Final volume	10	10	10

*Amounts added depended on molar ratios of cDNA to vector of 3:1 and 1:3. The calculation followed the formula: ng insert=(ng vector × kb size of insert)/kb size of vector × insert:vector ratio.

E. coli DH5α cells from a frozen glycerol stock were plated using a loop onto a new LB ampicillin plate (Appendix 7) and the plate was incubated at 37 °C overnight. A single colony of DH5α was transferred to a 25 ml sterilin with 10 ml LB (Appendix 6) and incubated at 37 °C on an orbital shaker at 150 ×g overnight. The following day 1 ml of cultured cells was transferred to a pre-autoclaved 1 litre flask with 100 ml LB. The flask was incubated at 37 °C on an orbital shaker at 150 ×g for about 2-3 hrs until OD_{550} equals 0.4. The flask was then taken from the shaker and put on ice for 20 min. The cultured cell solution was poured into 4 sterilins, each containing about 25 ml of cell solution. The sterilins were put on ice and were centrifuged at 300 ×g (4 °C) for 7 min. The supernatant was discarded and the tubes were placed upside down on tissue paper for about 1 min and then kept on ice. Pre-cooled 0.1 M $MgCl_2$ solution (5 ml) was added to each tube and resuspended by pipette. The tubes were centrifuged at 3,000 ×g (4 °C) for 7 min and the supernatant was discarded. Pre-cooled 0.1 M $CaCl_2$ solution (1 ml) was added to the first tube and the cells were gently resuspended by a pipette. The resuspended cells were transferred to the second tube and the cells were gently resuspended by a pipette, the same procedure was applied to the remaining tubes. The last tube had all resuspended cells and was kept on ice for a minimum of 90 min.

Transformation was carried out by adding 10 µl of diluted ligation solution, which was made adding 10 µl dH_2O to 10 µl ligation solution, to 200 µl of the competent cells in a 1.5 ml Eppendorf tube, and mixing gently. The tube was placed on ice for 20 min prior to heat shocking the cells for 2 min in a water bath at exactly 42°C. Afterwards the tube was put on ice immediately for 2 min, and 1 ml SOC solution (Appendix 9) (room temperature) was added to the tube, mixed gently. The tube was then incubated

for 1.5 hr at 37 °C without shaking. Three volumes (100, 200 and 300 µl) of each transformation solution were plated onto LB ampicillin/IPTG/X-Gal plates (Appendix 8) separately, these plates were incubated overnight at 37 °C and then stored at 4 °C.

3.2.8 Characterisation of cloned fragments by restriction analysis

About 30 white colonies of each band were transferred to a new LB/ampicillin/X-Gal plates using the following procedure. A grid was made on the bottom of fresh LB/ampicillin/X-Gal plates with a marker pen and each square was numbered. The plates with colonies together with fresh plates were put in a microfuge cabinet for about 30 min before white colonies were transferred to new LB/ampicillin/X-Gal plates. Plates were incubated overnight at 37 °C and maintained at 4 °C until required. Colonies were transferred to fresh LB/ampicillin/X-Gal plates once a month.

From the colony library plate of each differential band 10 to 15 colonies were chosen and each colony was transferred to a sterilin tube containing 5 ml sterilized LB and 5 µl ampicillin stock solution (50 mg/ml). Tubes were incubated overnight at 37°C in a shaker at 150 ×g, the cell solution of 1.5 ml was transferred to an Eppendorf and spun down at 12,000 ×g for 1 min. The supernatant was discarded before 150 µl of STET buffer (Appendix 17) was added to each tube prior to vortexing. After adding 8 µl of lysozyme/RNase solution (Appendix 18), tubes were incubated for 10 min at room temperature followed by boiling in a water bath for 40 seconds. After cooling down on ice, the tubes were centrifuged immediately at 12,000 ×g for 10 min. The pellet was discarded and an equal volume of isopropanol was added to the supernatant and mixed well prior to storage at -20 °C for 60 min. The tubes were centrifuged at 12,000 ×g for 5 min at 4 °C, the supernatant was removed followed by addition of 300 µl of 100 %

ethanol to wash the pellet before tubes were centrifuged at 12,000 ×g for 2 min. The supernatant was carefully removed and the pellets were allowed to air dry. 36 µl dH_2O was added to resuspend the pellet by flicking and the tubes were stored at -20 °C prior to restriction enzyme digestion.

To screen the different size inserts of each differential band, plasmid DNA was digested with *Eco*R I. The basic protocol for all restriction enzyme digestion is given in Table 3.11. The mixture was incubated overnight at 37 °C. The digested samples were run on a 1 % agarose gel to visualise variation in the size of the inserts under UV light.

Table 3.11 Protocol for restriction enzyme digestion of STET plasmid DNA.

Component	Volume (µl)
dH_2O	10.5
10 × enzyme buffer	2.0
BSA	0.5
Plasmid sample	6.0
EcoRI (10 unit/µl)	1.0
Total volume	20

3.2.9 Southern (slot) blot analysis

RT-PCR samples (20 µl) were loaded onto a 1 % agarose gel. After electrophoresis, the gel was transferred to a glass baking dish while unused areas of the gel were trimmed with a razor blade and the bottom left-hand corner of the gel was cut off. The DNA was denatured by soaking the gel for 45 min in several volumes of 1.5 M NaCl and 0.5 M NaOH with constant and gentle agitation. The gel was then briefly rinsed in dH_2O and neutralized by soaking in several volumes of a neutralisation solution containing 1 M Tris (pH7.4) and 1.5 M NaCl at room temperature for 30 min with constant and gentle agitation followed by a further 15 min with fresh neutralisation solution. Meanwhile, a piece of Whatman 3 MM paper was covered on a stack of glass plates to form a support that was longer and wider than the gel and was placed in a large baking dish. The dish was then filled with transfer buffer (10×SSC) (Appendix 10) until the level of the liquid reached almost to the top of the support. When the 3MM paper on the top of the support was thoroughly wet, air bubbles were carefully smoothed out with a glass rod. Nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was cut 1 mm larger than the gel in both dimensions and cut in one corner for orientation. The membrane was floated on the surface of the dH_2O until it was completely wet, and then was immersed in transfer buffer (10×SSC) for at least 5 min. The gel was removed from the neutralisation solution and placed inversely on the centre of the support, air bubbles between the gel and 3MM paper were smoothed out. Some parafilm was used to cover the surrounding area of the support and the membrane was placed on top of the gel aligning the cut corners, air bubbles between the gel and membrane were also smoothed out. Two pieces of 3MM paper that were cut to the same size as the gel and immersed in 2×SSC solution were placed on top of the membrane and air bubbles were smoothed out with

a glass rod. A stack of paper towels (10 cm high) were cut just smaller than the 3MM papers and placed on the 3MM papers. A glass plate was placed on top of the stack and weighed down with a 500 g weight. After 24 hrs, the paper towels and the 3MM papers were removed and the nylon membrane was soaked in 6×SSC for 5 min at room temperature. The membrane was placed on a paper towel to dry for 30 min at room temperature. Afterwards, it was sandwiched between two sheets of 3MM paper and baked for 2 hrs at 80 °C. The membrane was stored at -20 °C.

Double PCR with SP6 and T7 primers (SP6: 5'-TAT TTA GGT GAC ACT ATA G-3'; T7: 5'-TAA TAC GAC TCA CTA TAG GG-3') to amplify the selected pGEM-T plasmid DNAs was carried out using the following protocol (Table 3.12) and the following components (Table 3.13).

Table 3.12 PCR protocol for DNA probes.

Programme	Temperature (°C)	Time (min)	Cycle
1*	95	5	1
2	94	1	45
	49	2	
	72	2	
3	72	10	1

*After programme 1, 0.5 µl of DyNAzyme *Taq* DNA polymerase was added to the PCR mixture

Table 3.13 Components of PCR mixture for probes.

Reagent	Volume added (µl)
10×DNA Polymerase reaction buffer (with 15 mM $MgCl_2$)	5
dNTP mixture solution (5 mM of each dNTP)	2
Template	1 (plasmid DNA) or 2 (PCR product)
SP6 primer (20 pmole)	1
T7 primer (20 pmole)	1
DyNAzyme^{EXT} Taq DNA polymerase (1 unit/ 1µl) (Flogen)	0.5
dH_2O	Make up to 50 µl (final volume)
Mineral oil	50

The double PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions (3.2.7). The purified probes (10 µl) were loaded on a 1 % agarose gel to check purity and concentration while the remaining of the probe solutions were stored at -20 °C for labelling.

Radioactive probes were made using a Prime-a-Gene kit (Promega, Madison, WI, USA). Approximately 50 ng of template DNA was made up to 10 µl with nuclease free water and denatured by boiling for 2 min in a water bath, and then it was immediately chilled on ice. The reaction components were added into a 0.2 ml sterilised Eppendorf following the order in Table 3.14.

Table 3.14 Components for probe labelling with ^{32}P.

Components	Volume
Nuclease free water	29 µl
5x labelling buffer	10 µl
Unlabelled dNTPs mixture	2 µl
Denatured DNA	5 µl
Nuclease free BSA	2 µl
^{32}P-dATP (10 µCi)	1 µl
DNA polymerase I (5 units)	1 µl

Before adding ^{32}P-dATP, the tubes were moved into a radioactivity protection cabinet. After adding ^{32}P-dATP and DNA polymerase I, the mixture was homogenised with a pipette and incubated for 60 min at room temperature. The reaction was terminated by boiling for 2 min followed by cooling on ice. These labelled probes were either used directly or stored at -20 °C.

The nylon membrane with RT-PCR products was immersed in 5×SSC solution for about 1 min and fitted into a pre-warmed hybridisation tube (65 °C for 30 min). 10-15 ml of hybridisation solution (0.2 ml/cm^2 filter) consisting of 6×SSC, 0.5 % SDS (Appendix 11), 100 µg/ml denatured fragmented salmon sperm DNA (produced by immersion in a boiling water bath for 5 min followed by 2 min on ice) and 5×Denhardt's reagent (Appendix 12). The tube was continuously run at 65 °C for 1 hr. Meanwhile, the ^{32}P-labelled probe was denatured by boiling for 5 min followed by

rapidly chilling on ice and briefly centrifuging. The denatured probe was added to the tube and the tube was continuously run at 65 °C overnight.

The hybridisation solution was poured out into a container after which the filter was removed and immediately submerged in a tray containing 300 ml of 2×SSC and 0.5 % SDS at room temperature. After 5 min, the filter was transferred to a fresh tray containing 300 ml of 2×SSC and 0.1 % SDS, and incubated for 15 min at room temperature with occasional gentle agitation. The filter was transferred to a flat-bottom plastic box containing 300 ml of fresh 0.1×SSC and 0.5 % SDS. The filter was incubated for 30 min at 37°C with gentle agitation. The box was transferred to a water bath set at 68 °C with 300 ml of fresh 0.1×SSC and 0.5 % SDS and incubated for a further 30 min. The filter was briefly washed with 0.1×SSC at room temperature, most of the liquid was removed from the filter by placing it on a pad of paper towels. The damp filter was placed on a sheet of Saran Wrap and exposed to X-ray film at -70 °C.

3.2.10 Northern blot analysis

A 0.7 % agarose gel, was prepared in DEPC treated water. When it was cooled to 60 °C, 30 ml of 5×formaldehyde gel-running buffer [0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS), 40 mM sodium acetate and 5 mM EDTA (pH 8.0)] and 27 ml of formaldehyde were added to give final concentrations of 1× formaldehyde gel-running buffer and 2.2 M formaldehyde. The gel was cast in a chemical hood and allowed to set for at least 30 min at room temperature. Meanwhile, the RNA sample was prepared as in Table 3.15.

Table 3.15 Composition of RNA sample for electrophoresis.

Component	Volume
RNA (30 μg)	4.5 μl
5×formaldehyde gel-running buffer	2.0 μl
formaldehyde	3.5 μl
formamide	10.0 μl

Before loading on the gel, the mixture was incubated at 65 °C for 15 min, chilled on ice and centrifuged for 1 min. Then 2 μl of sterile and DEPC-treated formaldehyde-gel loading buffer (Appendix 13) was added and mixed. After pre-running the gel for 5 min at 5 V/cm, the samples and molecular weight markers were immediately loaded on the gel and the gel was run overnight at 3-4 V/cm in 1×formaldehyde gel-running buffer. At the end of the run (when the bromophenol blue band had migrated approximately 8 cm), the lane containing the marker was cut from the gel and stained by immersing in 0.1 M ammonium acetate solution with 0.5 μg/ml ethidium bromide for 60 min. Aligning a transparent ruler with the gel, a photograph was taken and it was used to measure the distance from the loading well to each of the bands of RNA. A plot of the $\log_{10}$ of the size of the fragments of RNA against the distance migrated was utilized to calculate the size of the RNA species detected by hybridisation.

The gel was rinsed in DEPC-treated water 3 times to remove the formaldehyde, and soaked in 0.05 M NaOH for 20 min. The gel was then rinsed in RNase free water prior to soaking in DEPC-treated 20×SSC for 45 min. The rest of operations were similar to

3.2.9 except using 20×SSC instead of 10×SSC and all solutions and equipment were treated with DEPC and sterilised.

The RNA nylon membrane was immersed in DEPC-treated 5×SSC solution for 2 min and then fitted into a pre-warmed hybridisation tube (42 °C for 30 min). 10-15 ml of hybridisation solution (0.2 ml/cm^2 filter), which included: 50 % formamide, 5×SSPE (Appendix 14), 0.1 % SDS (Appendix 11), 100 μg/ml denatured fragmented salmon sperm DNA and 2×Denhardt's reagent (Appendix 12). The tube was continuously run at 42 °C for 1-2 hr. Meanwhile, the ^{32}P-labelled probe (3.2.9) was denatured by boiling for 5 min followed by rapidly chilling on ice and briefly centrifuged. The denatured probe was added to the tube and the tube was continuously run at 42 °C overnight.

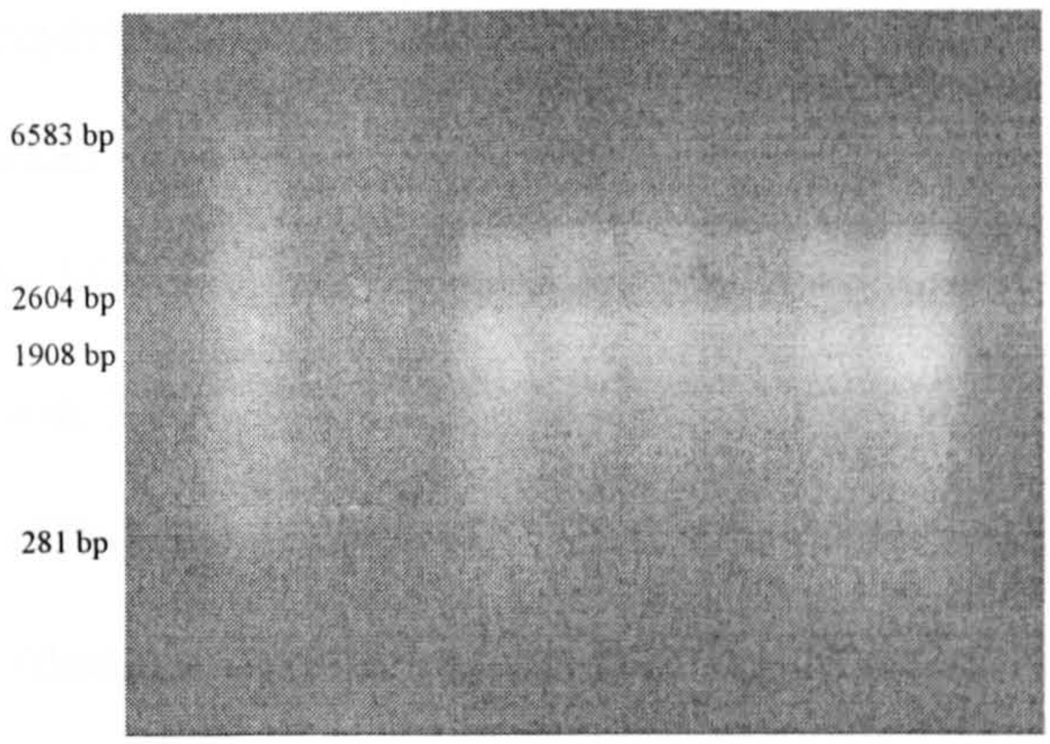
After that the hybridisation solution was poured into a container. The filter was removed and immediately submerged in a tray containing 300 ml of DEPC-treated 1×SSC and 0.1 % SDS at room temperature for 20 min. This was then followed by 3 washes of 20 min each at 68 °C in 200 ml of 0.2×SSC and 0.1 % SDS. Most of the liquid was removed from the filter by placing it on a pad of paper towels and the damp filter was placed on a sheet of Saran Wrap and exposed to X-ray film at -70 °C.

3.3 Results

3.3.1 Total RNA extraction

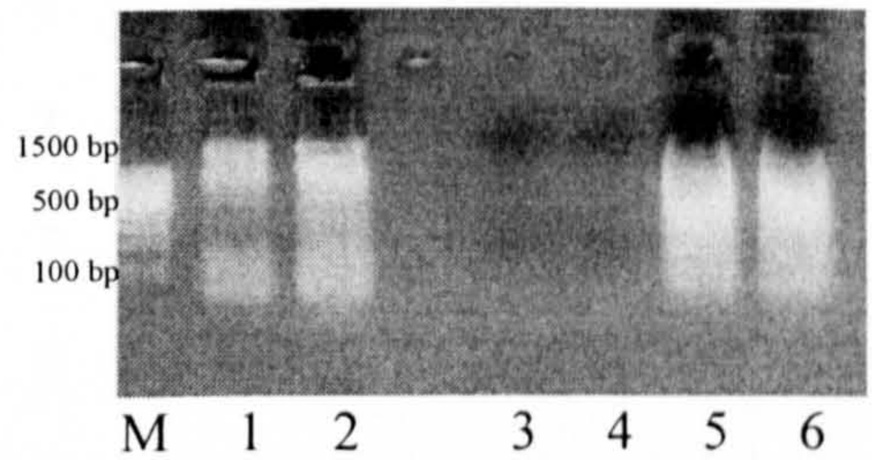
RNA was successfully extracted from all samples and high A_{260}/A_{280} ratios indicated low protein contamination (Appendix 15). The extracted RNA size is shown in Fig. 3.2, the size of extracted RNA is approximately from 2.0 kb and 0.1 kb (lanes 2 - 7). The rRNA band is on the top of each lane of the samples and the size is between 3.5 kb and 2.5 kb. The extracted RNA quality was checked by digestion with DNase and RNase (Fig. 3.3), there were some DNA residues in the extracted RNA sample without DNase digestion (lanes 2 and 3) compared to the same samples treated by RNase-free DNase (lanes 6 and 7). No signal was observed after the samples were digested with both DNase and RNase (lanes 4 and 5) indicating that RNase had broken down any RNA present.

Fig. 3.2 Electrophoresis of RNA on 0.7 % agarose formaldehyde gel.



Lane	sample	Lane	Sample
M	RNA marker	4	MAY-LTSS
1	GP-CK	5	GP-STSS
2	MAY-CK	6	MAY-STSS
3	GP-LTSS		

Fig. 3.3 RNA samples of recovery treatment leaves of Golden Promise and Maythorpe.



Lane	Sample
M	5 µl 100bp DNA ladder
1	MAY-Rec (Before DNase digestion)
2	GP-Rec (Before DNase digestion)
3	MAY-Rec (DNase + RNase digestion)
4	GP-Rec (DNase + RNase digestion)
5	MAY-Rec (After DNase digestion)
6	GP-Rec After DNase digestion

3.3.2 RT-PCR condition experiments

3.3.2.1 Initial RT-PCR experiment

The first RT-PCR experiment was carried out to ensure that the RT reaction and subsequent PCR worked properly. The RT reaction was performed as described in section 3.2.3 using the Maythorpe leaf control RNA sample. The subsequent PCR was performed as in section 3.2.4 with 6 different random/oligo-dT primer pairs (Table 3.16). No amplified cDNA bands were visible for all primer combinations when run on a 1 % agarose gel (data not shown).

Table 3.16 The initial PCR reaction components with Maythorpe control leaf RNA.

$MgCl_2$	10× buffer	dNTPs 5 mM	Poly-T primer	Random primer	RT-product	dH_2O	*Taq* 1.25 U
			µl				
3	5	2	1 (PT1)	1 (R1)	20	16	2
3	5	2	1 (PT1)	1 (R2)	20	16	2
3	5	2	1 (PT2)	1 (R1)	20	16	2
3	5	2	1 (PT2)	1 (R2)	20	16	2
3	5	2	1 (PT3)	1 (R1)	20	16	2
3	5	2	1 (PT3)	1 (R2)	20	16	2

Possible reasons for the failure could be from the RT reaction, such as inappropriate primers, poor enzyme quality or poor quality of dNTPs or from the PCR step such as incorrect Mg^{2+} concentration, unsuitable annealing temperature, sub-optimal template concentration and insufficient PCR cycles. Further PCR by changing the annealing temperature and the $MgCl_2$ concentrations (1.0, 1.5 and 2.0 mM) were also unsuccessful (data not shown).

3.3.2.2 A controlled PCR to check *Taq* polymerase activity and dNTPs

Amplification of an *A. nidulans agaA* fragment of the expected size (1.25 kb) from plasmid pAGA with specific primers was successfully performed (Fig. 3.4). This indicated that the *Taq* polymerase (Promega, Madison, WI, USA), reaction buffer and dNTPs were all functioning well. The failure to produce PCR products from barley templates therefore might be related to the reverse transcriptase reaction.

3.3.2.3 RT-PCR of garlic RNA using two-base anchored oligo-dT primers

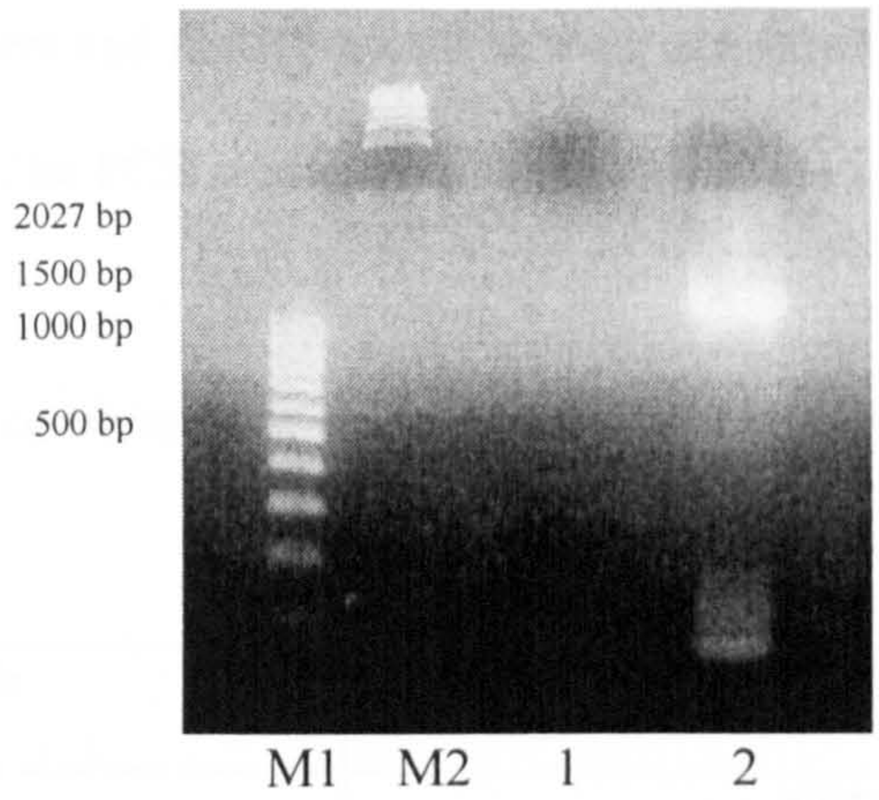
The reason to change one-base anchored oligo-dT primers to two-base anchored oligo-dT primers (Table 3.2) because the latter worked well with garlic plant RNA (Wei *et al.*, 2002a, in press) and it was expected that it can easily bind to the reading frame of poly-A tail of mRNA transcripts (Liang and Pardee, 1992). The other concern is that one-base anchored oligo-dT primer might bind nonspecifically anywhere within the poly A tail of the mRNA transcript and thus RT would not be successfully creating cDNA transcripts of the coding region of mRNA present in the sample. To test two-base oligo-dT primers, garlic shoot RNA was prepared with the same protocol as in section 3.2.2. RT was carried out with PT4, PT5 and PT6. PCR was conducted with primer combinations showed in Table 3.17.

Table 3.17 Primer combinations and PCR conditions for amplification of RT product of garlic mRNA.

Primer combination	$MgCl_2$	RT-product (µl)	Taq (unit)
PT4/ R2	1.5 mM	20	1.25
PT4/ R3	1.5 mM	20	1.25
PT5/ R2	1.5 mM	20	1.25
PT5/ R3	1.5 mM	20	1.25
PT6/ R2	1.5 mM	20	1.25
PT6/ R3	1.5 mM	20	1.25

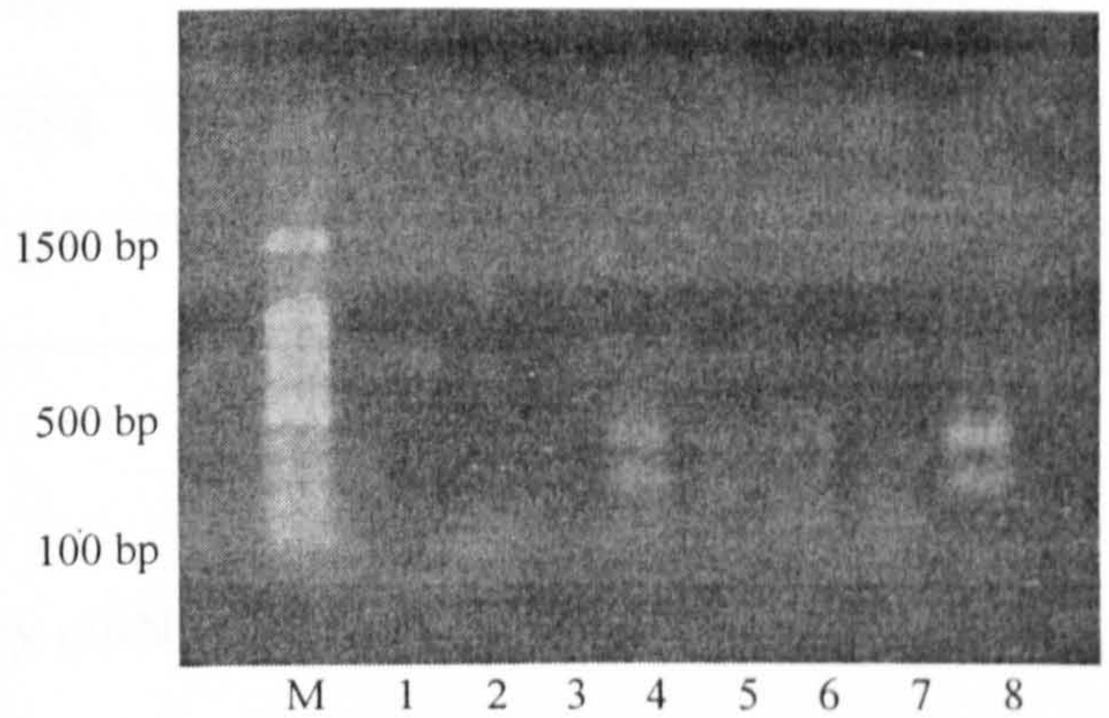
The resulting PCR products were run on a 1 % agarose gel (Fig. 3.5). Both negative and positive controls worked well, in lane 2 the signal indicated no DNA contamination, and in lane 3 the positive control DNA (*E. coli* DNA) was successfully amplified even though the yield was low. Fig. 3.5 also showed that RT-PCR of garlic shoot was successful, most of the primer combinations worked well except PT4/R2 (lane 3). The highest yield was obtained from primer combinations of PT4/R3 (lane 4) and PT6/R3 (lane 8). The size range of visible amplified products was between approximately 500 bp and 100 bp. The results indicated that RT and PCR reactions with the two-base anchored oligo-dT primers worked well with the garlic RNA sample and that the primer combination was crucial to get a high yield of RT-PCR products.

Fig. 3.4 Gel showing successful amplification of the *A. nidulans agaA* sequence from the plasmid pAGA.



Lane	Sample
M1	5 µl 100 bp DNA ladder
M2	5 µl λ *Hind*III DNA marker
1	0.02 µg plasmid pAGA DNA without PCR
2	10 µl PCR-product

Fig. 3.5 RT-PCR results of garlic shoot mRNA visualised on a 1 % agarose gel. All lanes except the marker contained 10 µl.



Lane	Sample	Lane	Sample
M	5 µl 100 bp DNA ladder	5	PT5/R2
1	Negative control without template without DNA template	6	PT5/R3
2	Positive control with *E. coli* DNA	7	PT6/R2
3	PT4/R2	8	PT6/R3
4	PT4/R3		

3.3.2.4 RT-PCR experiments on barley samples

Maythorpe leaf control mRNA sample was reverse transcribed with PT4, PT5 and PT6 poly-T anchored primers and the RT products were amplified by PCR using the oligo-dT anchored primers. The PCR primer combinations are shown in Table 3.18.

Table 3.18 Primer combinations used to amplify RT products of Maythorpe leaf control mRNA.

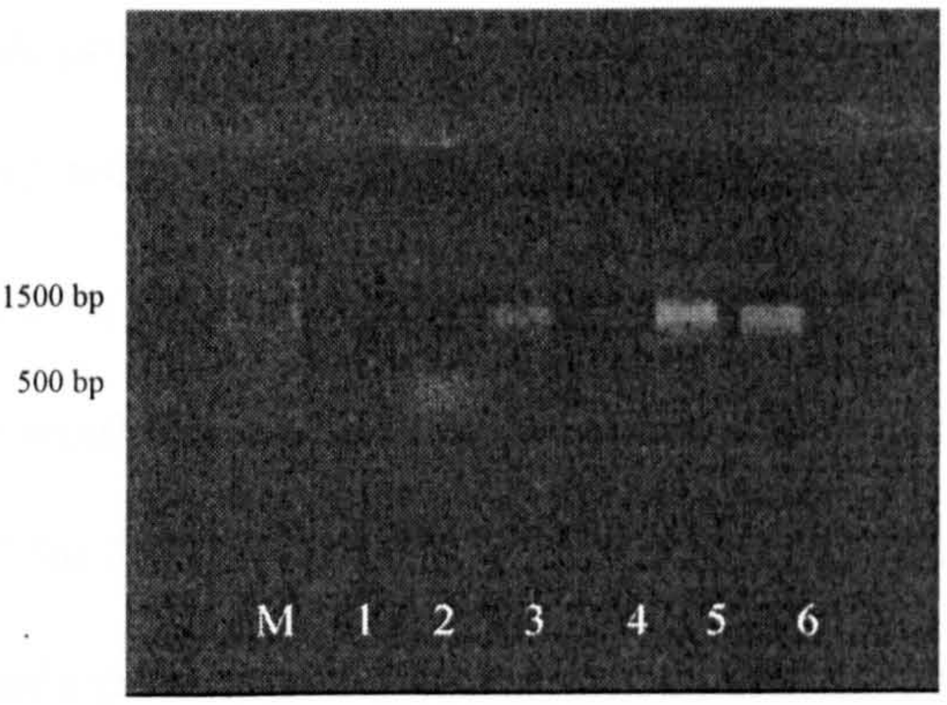
Sample	Primers
BP1	pT4/R1
BP2	pT4/R3
BP3	pT4/R4
BP4	pT4/R5
BP5	R3/R4
BP6	pT5/R1
BP7	pT5/R3
BP8	pT5/R4
BP9	pT5/R5

The PCR of barley mRNA samples was successful this time even though the signals on a 1 % agarose gel were weak (data not shown). The size range was approximately between 600 bp and 100 bp. All the above primer combinations worked while some had comparatively stronger signals than others. The low efficiency of PCR could be affected by reaction conditions, such as $MgCl_2$ concentration. Further experimentation was required to optimise PCR conditions in order to obtain better yield.

In order to produce high yields of cDNA from barley mRNA samples, the $MgCl_2$ concentration was altered from 1.5 mM to 2.5 mM in the PCR reaction solution and 50 μl mineral oil was overlaid on the solution. The results in Fig. 3.6 showed that the PCR of Maythorpe leaf control mRNA sample with 2.5 mM $MgCl_2$ produced the highest yield (lane 6) and 2.0 mM $MgCl_2$ also gave a good yield (lane 7).

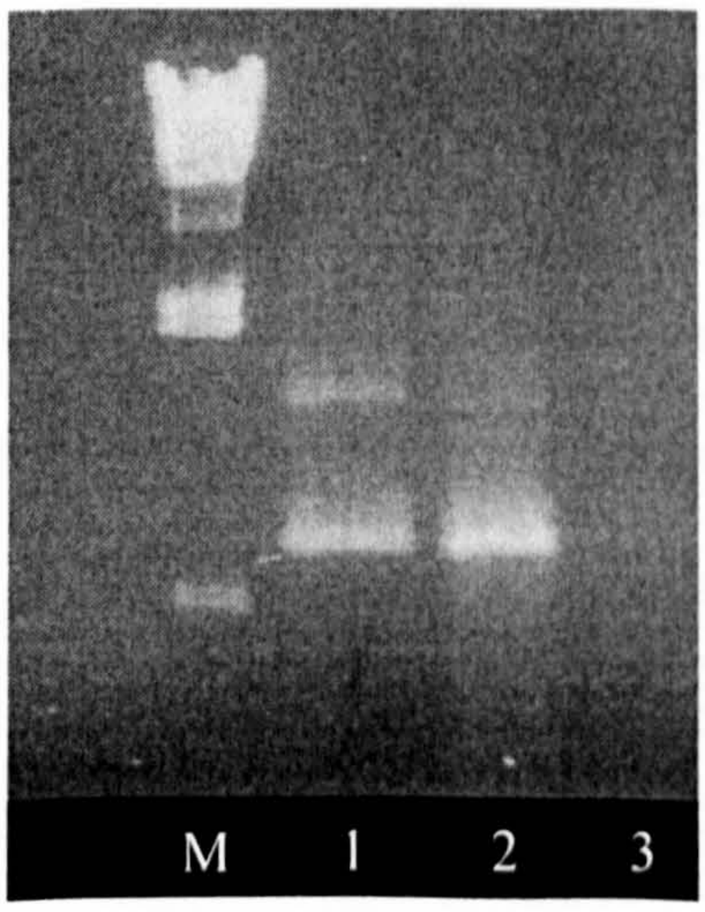
DNA polymerase is another key factor that affects PCR efficiency. Two DNA polymerases were compared in amplifying *E. coli* DNA, *Taq* DNA polymerase from Promega (Madison, WI, USA) and DyNAzyme^{EXT} from Flowgen (Lichfield, UK). The PCR reactions were carried out with same primer combination of PT4/R3 and the reaction conditions were the same as described in section 3.2.4. The results showed that both polymerases worked successfully (Fig. 3.7), but 1 unit of DyNAzyme with reaction buffer containing 1.5 mM $MgCl_2$ produced higher yields (lane 2) than 1.25 U of the *Taq* from Promega (Madison, WI, USA) with 2.5 mM $MgCl_2$ (lane 1). Therefore DyNAzyme^{EXT} from Flowgen (Lichfield, UK) was used in the following experiments.

Fig. 3.6 PCR results of RT product of Maythorpe leaf control mRNA with different $MgCl_2$ concentrations, the same primer combination of pT4/R3 was used for all the samples.



Lane	Sample	Load	Lane	Sample	Load
M	100 bp DNA ladder	5 µl	4		
1	Negtive control	30 µl	5	2.5 mM $MgCl_2$	30 µl
2	Positive control with *E. coli* DNA, 1.5 mM $MgCl_2$	30 µl	6	2.0 mM $MgCl_2$	30 µl
3	1.5 mM $MgCl_2$	30 µl			

Fig. 3.7 PCR products with different *Taq* DNA polymerases visulised on a 1 % agarose gel. Lane M: 5 µl of λ *Hind*III DNA marker; lane 1: 10 µl PCR product with 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA) and 2.5 mM $MgCl_2$; lane 2: 10 µl PCR product with 1 U of DyNAzyme (Flowgen, Lichfield, UK) with reaction buffer containing 1.5 mM $MgCl_2$; lane 3: negative control without template DNA.



From the above optimisation studies, further RT-PCR reactions on barley mRNA samples would apply the following:

- Two-base anchor oligo poly-T primers in both RT and PCR reactions.
- Both *Taq* DNA polymerases from Promega (Madison, WI, USA) and Flowgen (Lichfield, UK) were suitable in PCR of barley mRNA. Using the *Taq* from Promega (Madison, WI, USA), $MgCl_2$ should be adjusted to 2.5 mM.
- PCR reactions would be carried out for 35 cycles of 94 °C for 1 min, 40 °C for 2 min and 72 °C for 2 min with a final extension step of 10 min at 72 °C. Hot start at 95 °C for 5 min prior to addition of *Taq* polymerase would be applied.
- Mineral oil would be used to cover PCR reaction solution.

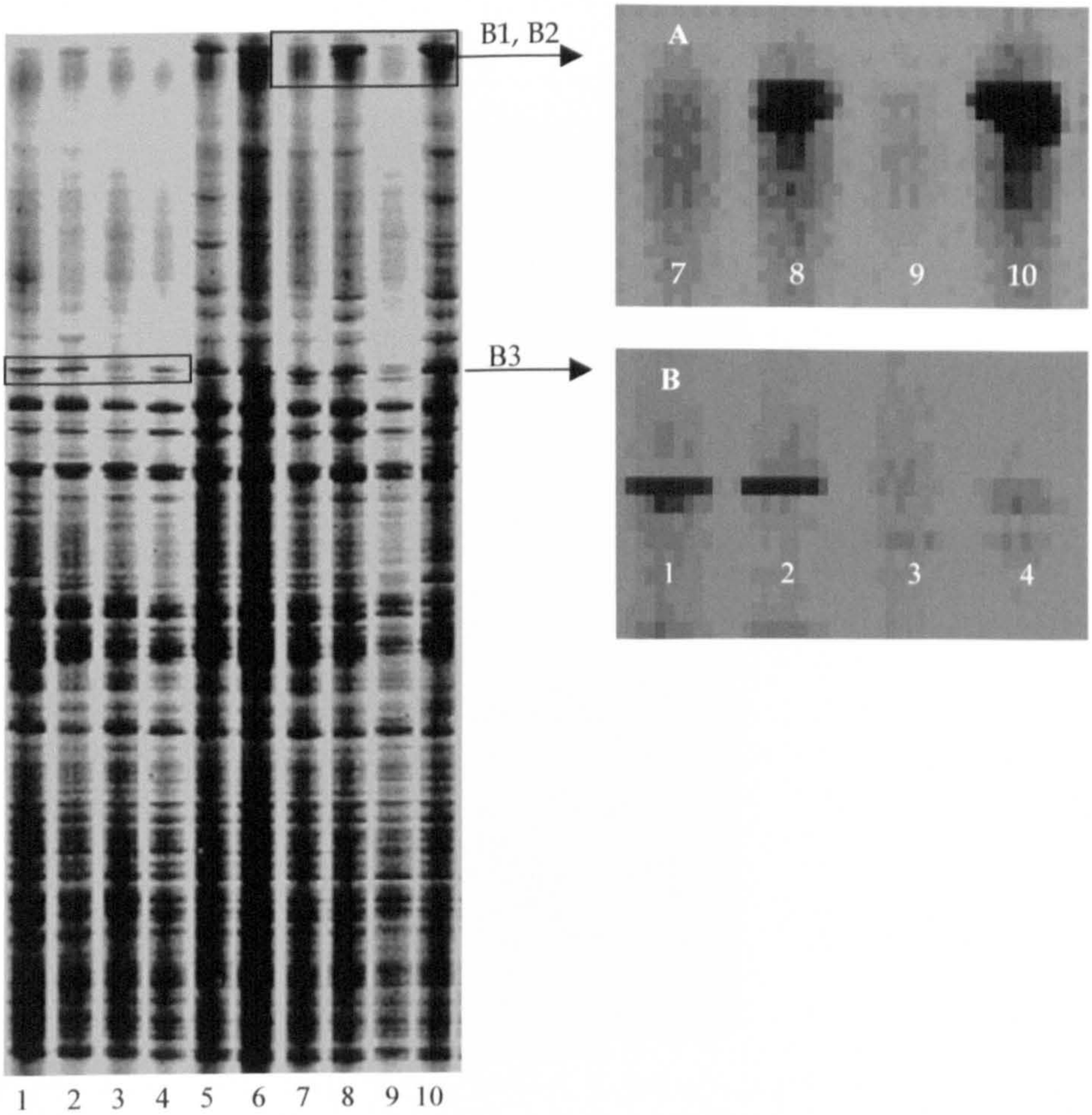
3.3.3 Separation of cDNA

RT-PCR products of both leaves and roots of Golden Promise and Maythorpe with different primer combinations were separated by polyacrylamide gels (section 3.2.6). About 135 cDNA fragments were amplified with leaf mRNA samples of either Golden Promise or Maythorpe by using the primer combination of PT4/R3 (Fig. 3.8), but only 3 differential fragments were identified, which were labelled as B1, B2 and B3. B1 and B2 did not separate well as can be seen clearly in box A. These two fragments showed strong signals in leaves from the short-term salt stress (STSS) (lanes 8 and 10) and recovery treatments (Rec) (lanes 5 and 6) of both Golden Promise and Maythorpe. B3 showed a strong signal in Maythorpe under control conditions (MAY-CK, lanes 1 and 7), short-term salt stress (MAY-STSS, lane 8), long-term salt stress (MAY-LTSS, lane 2) and recovery treatment (MAY-Rec, lane 5), it also showed strong signal in the Golden Promise short-term salt stress (GP-STSS, lane 10) and recovery treatments (GP-Rec, lane 6). The major differences between the two cultivars in B3 were found

under control conditions and long-term salt stress where Maythorpe showed a much stronger signal than Golden Promise. The same procedures were applied to the other polyacrylamide gels using different primer combinations.

More differential bands were identified from the primer combination PT6/R5 (Fig. 3.9). Differential bands B15, B17, B19 and B20 were differentially amplified in GP-CK and B27, B16, B18 and B22 in MAY-CK treatments, respectively. Bands B8, B9 and B11 were identified from the short-term salt stress (STSS) in both Golden Promise and Maythorpe leaves while B12 was selected from MAY-STSS, MAY-BT and MAY-MS treatments. B13 was induced by general stress, *i.e.* salt, boron toxicity and mannitol in both cultivars.

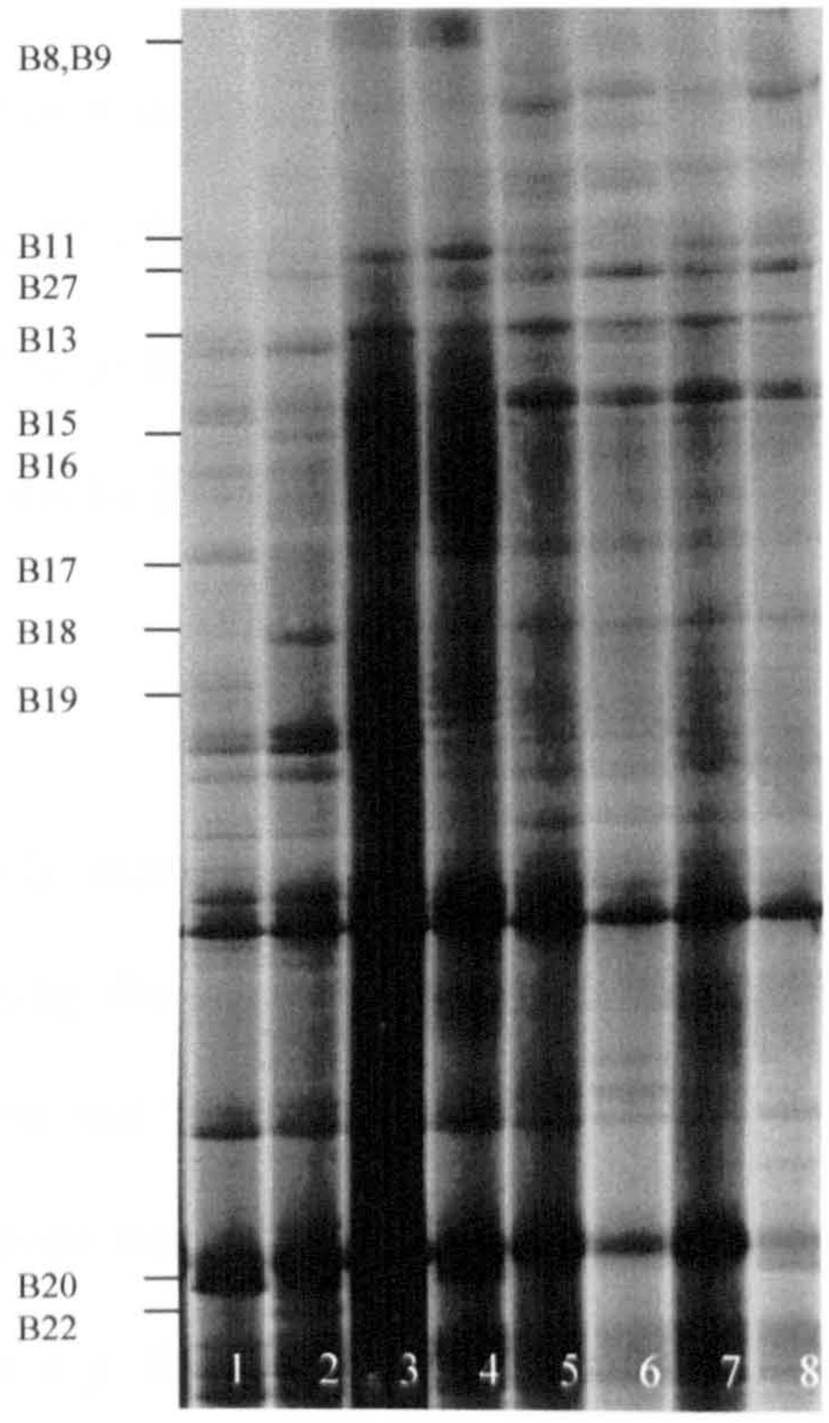
Fig. 3.8 Autoradiography of polyacrylamide gel with ^{35}S labelled PCR products of leaf mRNA. All samples used the same primer combination of PT4/R3.



Lane	Sample (leaf)	Lane	Sample (leaf)
1	MAY-CK	6	GP-Rec
2	MAY-LTSS	7	MAY-CK
3	GP-CK	8	MAY-STSS
4	GP-LTSS	9	GP-CK
5	MAY-Rec	10	GP-STSS

MAY: Maythorpe; GP: Golden Promise

Fig. 3.9 Autoradiography of polyacrylamide gel with ^{35}S labelled PCR products of leaf mRNA. All samples used the same primer combination of PT6/R5.



Lane	Sample (leaf)	Lane	Sample (leaf)
1	GP-CK	5	GP-BT
2	MAY-CK	6	MAY-BT
3	GP-STSS	7	GP-MS
4	MAY-STSS	8	MAY-MS

MAY: Maythorpe; GP: Golden Promise

With the primer combination of PT4/R4, four differential bands were detected (data not shown). Band B23 identified from short-term salt stress treatments in both Golden Promise and Maythorpe. Bands B24 and B25 were differentially amplified in the long-term salt stress leaves of Maythorpe. Band B26 was a differential band induced by mannitol stress in both cultivars.

RT-PCR was also performed with root mRNA samples of both plants using the primer combinations of PT4/R3, PT6/R5 and PT4/R4, but they did not produce enough bands. Most of the samples had poor signals (data not shown). Thus it was difficult to clearly identify any differential bands from the results such that leaf samples were used in all subsequent experiments.

A total of 28 differentially expressed cDNA transcripts from leaves in relation to salt stress were identified using the 3 primer combinations PT4/R3, PT6/R5 and PT4/R4 between Golden Promise and Maythorpe (Table 3.19). Some of these bands were associated with differences between the cultivars Golden Promise and Maythorpe under control conditions, *e.g.* B15, B16, B17, B18, B19, B20 and B22; some of them were salt inducible and were identified from short-term and long-term salt stress treatments, *e.g.* B8, B9, B28 and B23. Others were induced by ion toxicity and osmotic stress treatments, *e.g.* B4, B5, B6, B10 and B12; and some bands were differentially expressed in relation to general stress (salinity, ion toxicity and mannitol), *e.g.* B1, B2, B11 and B14.

Table 3.19 Differential cDNA bands of barley leaves with three primer combinations.

Differential band	Primer combination	Expression condition
B1	PT4/R3	STSS, BT, MS
B2	PT4/R3	STSS, BT, MS
B3	PT4/R3	MAY-CK, LTSS, STSS
B4	PT4/R3	BT, MS
B5	PT4/R3	BT, MS
B6	PT4/R3	BT, MS
B7	PT4/R3	MS
B8	PT6/R5	STSS
B9	PT6/R5	STSS
B10	PT6/R5	BT, MS
B11	PT6/R5	STSS, BT, MS
B12	PT6/R5	MAY-BT, MAY-MS
B13	PT6/R5	GP-STSS, BT, MS
B14	PT6/R5	STSS, BT, MS
B15	PT6/R5	GP-CK
B16	PT6/R5	MAY-CK
B17	PT6/R5	GP-CK
B18	PT6/R5	MAY-CK
B19	PT6/R5	GP-CK
B20	PT6/R5	GP-CK
B21	PT6/R5	STSS
B22	PT6/R5	MAY-CK
B23	PT4/R4	STSS
B24	PT4/R4	MAY-LTSS
B25	PT4/R4	MAY-LTSS
B26	PT4/R4	MS
B27	PT6/R5	MAY-CK
B28	PT6/R5	MAY-STSS

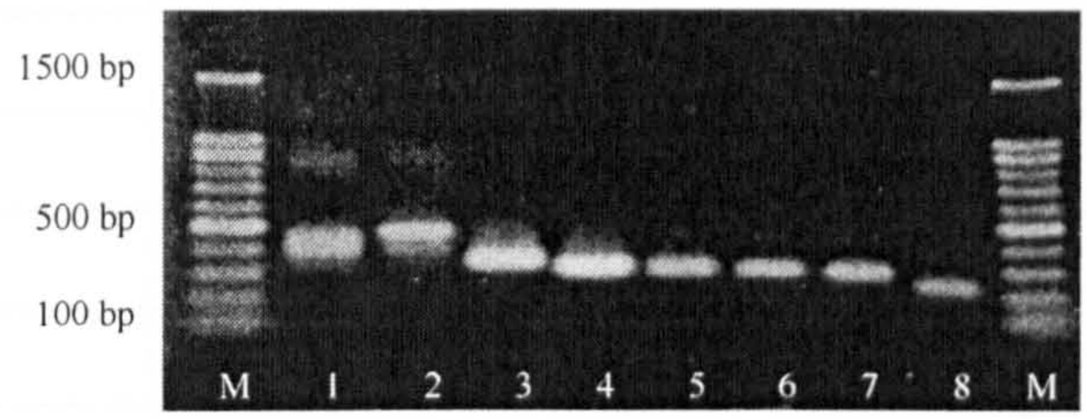
All of the differential bands identified were cut from the polyacrylamide gel for further analysis. It is very important to excise the bands accurately and so to confirm that interested bands were correctly excised, gels were exposed to X-ray film again after cutting. The results confirmed that all of the differential bands were excised accurately (data not shown).

3.3.4 Recovery and purification of cDNA transcripts

The differential cDNA fragments, which were removed from polyacrylamide gels were recovered as described in section 3.2.6 followed by PCR amplification using the same primer combinations as in differential display. Amplified bands following PCR are shown in Fig. 3.10. Some samples had one strong band and the size was between 200 bp and 500 bp (Fig 3.10a, c). Some samples e.g. B1, B2, B23, B26, B11 and B12 exhibited two amplified bands (Fig 3.10b), the size of the large band was between 500 bp and 800 bp and the size of the small bands was between 200 bp and 500 bp. It is possible that there are co-migrated fragments with differential bands and that some adjacent bands had also been excised from the polyacrylamide gels when in close proximity to the target band. Therefore, to identify true differential cDNA fragments from RT-PCR products, Southern (slot) blot analysis was carried out using RT-PCR products as template DNA sources and cloned differential fragments as probes. The probes were produced by cloning the excised and purified fragments into pGEM-T Easy vector (Promega, Madison, WI, USA) and transforming them into *E. coli* DH5α cells. The transformation results of five samples are shown in Table 3.20 as examples.

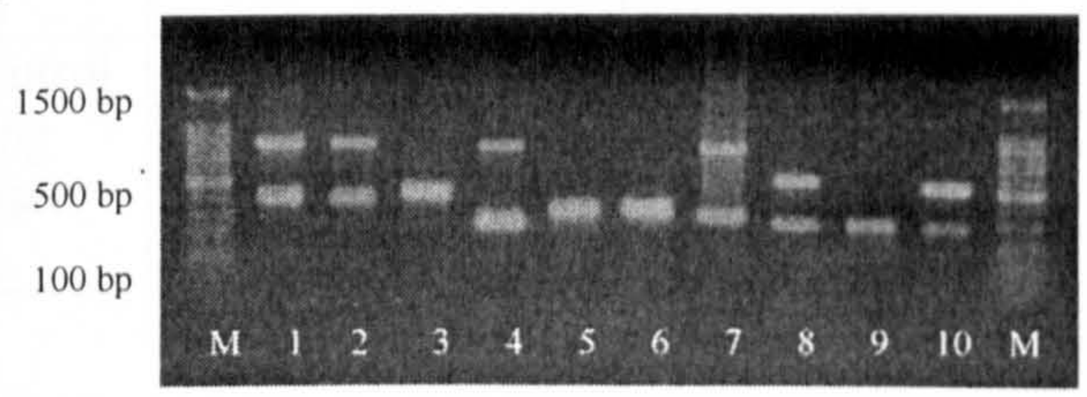
Fig. 3.10 Purified PCR products of differential bands excised from polyacrylamide gels visualised on a 1 % agarose gel. All samples were loaded with 10 µl and DNA marker was loaded with 5 µl. In b: B1*, B2* and B3* were excised from a different polyacrylamide gel.

a



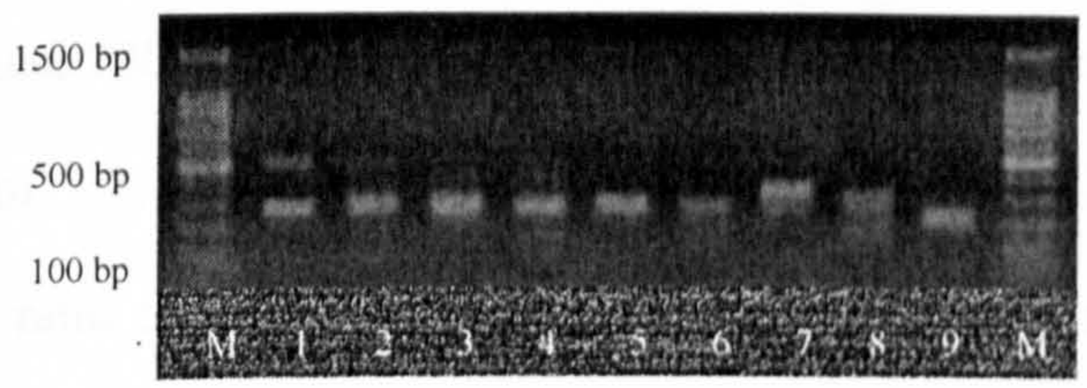
Lane	Sample	Lane	Sample
M	100 bp DNA ladder	5	B8
1	B1, B2	6	B9
2	B3	7	B21
3	B4	8	B20
4	B5	M	100 bp DNAladder

b



Lane	Sample	Lane	Sample
M	100 bp DNA ladder	6	B25
1	B1*	7	B26
2	B2*	8	B11
3	B3*	9	B27
4	B23	10	B12
5	B24	M	100 bp DNA ladder

c



Lane	Sample	Lane	Sample
M	100 bp DNA ladder	6	B17
1	B28	7	B18
2	B13	8	B19
3	B14	9	B22
4	B15	M	100 bp DNA ladder
5	B16		

Table 3.20 Colony counting of transformation of differential cDNA fragments. The counts presented are from plate with 300 μl of transformed cell solution.

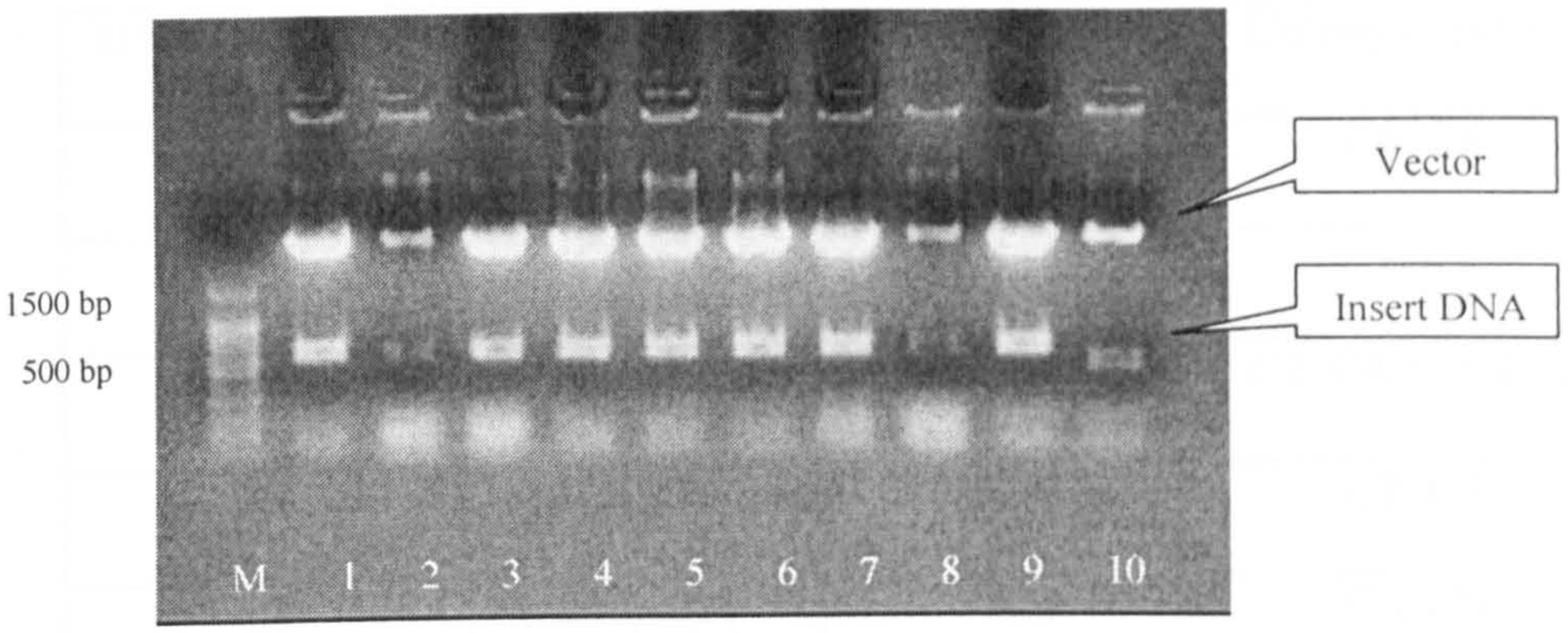
Sample	Ratio (template:vector)	White colonies	Blue colonies
B1, B2	3:1	45	9
	1:3	27	3
B3	3:1	100	48
	1:3	75	38
B8	3:1	59	8
	1:3	8	1
B9	3:1	9	0
	1:3	9	2
Negative control (without insert DNA)		0	3
Positive control (with 8 ng control insert DNA)		125	0

From Table 3.20 both controls worked well, there are no white colonies and 3 blue colonies in the negative control and 125 white colonies and no blue colonies in the positive control. The efficiency of transformation varied among different samples, for example, B3 had 100 white colonies but B9 just had 9 white colonies with template to vector ratio of 3:1. In comparison with the two template DNA to vector ratios, generally, the ratio of 3:1 produced more white colonies than the ratio of 1:3. All the excised bands were successfully cloned. After that, a colony library of each differential band was produced by transferring the white colonies to a new LB/ampicillin/X-Gal (Appendix 8). Each library was screened for different sizes of inserts by restriction enzyme digestion (*Eco*R I) of plasmid DNAs from 10 to 15 white

colonies. For example, the plasmid DNAs of ten white colonies of band B11 were digested with *Eco*R I (Fig. 3.11), the colonies from C1 to lane C9 in lane 1 to lane 9 had same size inserts of about 0.6 kb, C1 was chosen to represent these 9 colonies. Colony C13 in lane 10 had different size insert from others, which was about 0.5 kb. Therefore, C1 and C13 of band B11 were selected for further analysis. Another example is shown in Fig. 3.12 where 10 plasmid DNA samples from different colonies of band B13 were digested by *Eco*RI, the results showed that the inserts in lanes 1, 3, 8, 9 and 10 were of different size and they were therefore selected for further analysis.

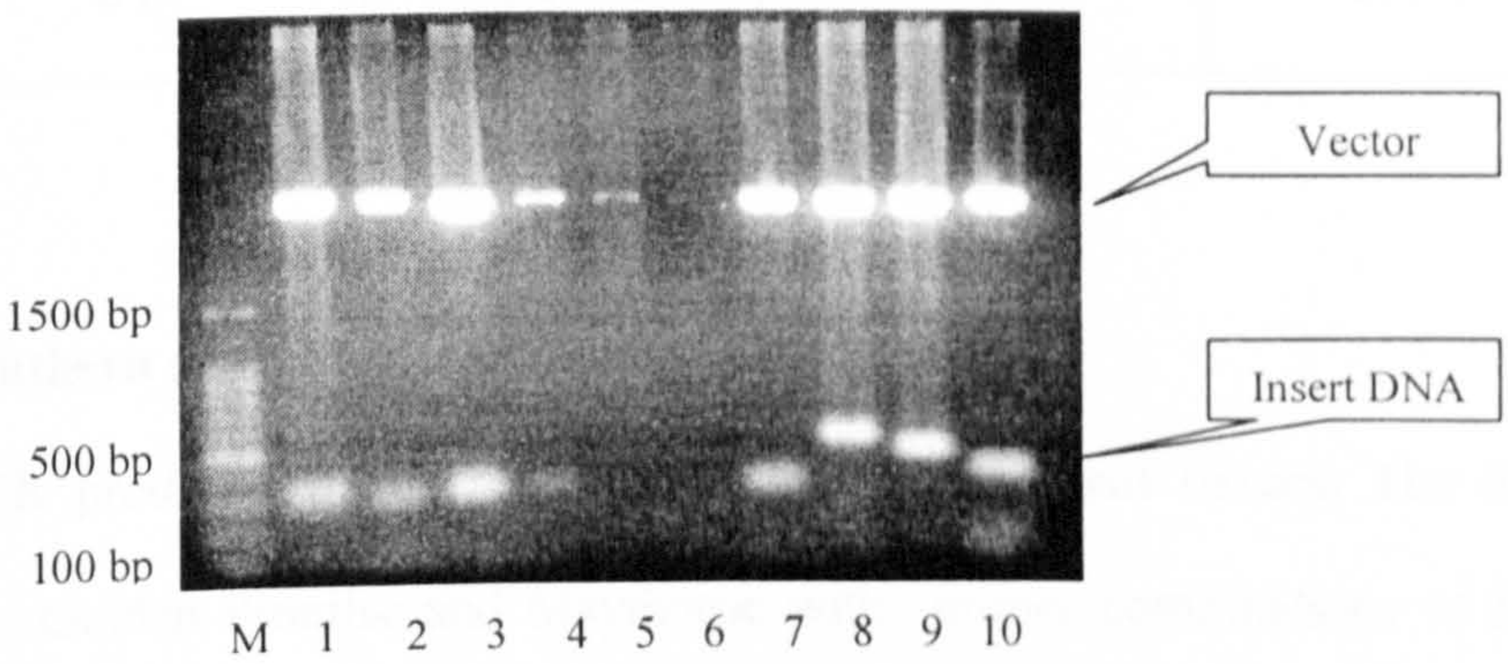
A second screening was carried out on all potential different inserts in size from 28 bands by running PCR with the SP6 and T7 primers. The confirmed different size inserts (Table 3.21) were used to develop probes for Southern (slot) blot analysis. Most differential bands produced clones with varied size inserts, for example, band B17 had 4 different size inserts whereas bands B11, B12, B28, B16 and B25 had just a single size insert.

Fig. 3.11 Plasmid DNA of B11 digested by *Eco*R I. The colonies were selected for further analysis showed in bold letters.



lane	1	2	3	4	5	6	7	8	9	10
colony	**C1**	C2	C3	C4	C5	C6	C7	C8	C9	**C13**

Fig. 3.12 Plasmid DNA of B13 digested by *Eco*R I. The colonies were selected for further analysis showed in bold letters.



lane	1	2	3	4	5	6	7	8	9	10
Colony	**C1**	C2	**C4**	C5	C6	C8	C17	**C18**	**C19**	**C20**

Table 3.21 The selected colonies with varied size inserts of the differential bands after PCR screening with SP6 and T7 primers.

Differential band	Colony number	Differential band	Colony number
B1, B2	C4, C19	B15	C6, C8
B3	C1, C4	B16	C2
B4	C2, C5	B17	C2, C4, C6, C12
B5	C5, C6	B18	C2, C3
B8	C13, C20	B19	C4, C9
B9	C2, C4	B20	C3, C10, C12
B11	C1	B21	C2, C10
B27	C4, C5	B22	C6, C11
B12	C1	B23	C6, C9
B28	C1	B24	C4, C11
B13	C4, C18, C19	B25	C1
B14	C3, C8, C9	B26	C2, C5

3.3.5 Southern (slot) blot analysis

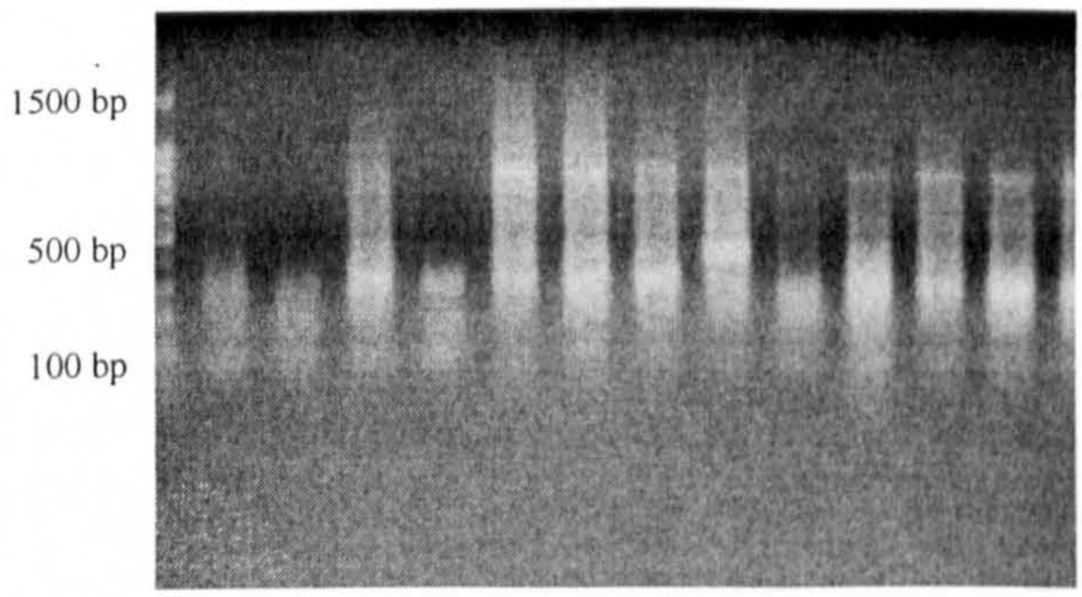
All RT-PCR products in this section are derived from leaf tissues. The RT-PCR products of Golden Promise and Maythorpe with primer combinations of PT4/R3, PT4/R4 and PT6/R5 were separately blotted to Hybond-N^+ nylon membranes (Amersham). With the primer combination PT4/R3 (Fig. 3.13), the size of amplified cDNA fragments of all the samples was between 100 bp and 600 bp. Lanes 5, 6 and 7 showed a more intense band at about 500 bp than the others. With the primer combination PT4/R4 (Fig. 3.14), the cDNA fragments covered a much wider range

between 100 and 1500 bp in size except for GP-CK, MAY-CK and GP-LTSS in which the size of visualised cDNA fragments was less than 500 bp. In the case of the PT6/R5 primers the PR-PCR products were evenly distributed among the samples (Fig. 3.15) and the size of cDNAs of all the samples was less than 500 bp.

Fig. 3.13 1 % agarose gel of RT-PCR products of barley leaves of Golden Promise and Maythorpe with the primer combination PT4/R3, all lanes were loaded 20 µl of sample.

Lane	Sample	Lane	Sample
M	5 µl 100bp ladder	7	GP-STSS
1	GP-CK	8	MAY-STSS
2	MAY-CK	9	GP-BT
3	GP-LTSS	10	MAY-BT
4	MAY-LTSS	11	GP-MS (shoot)
5	GP-Rec	12	MAY-MS (shoot)
6	MAY-Rec		

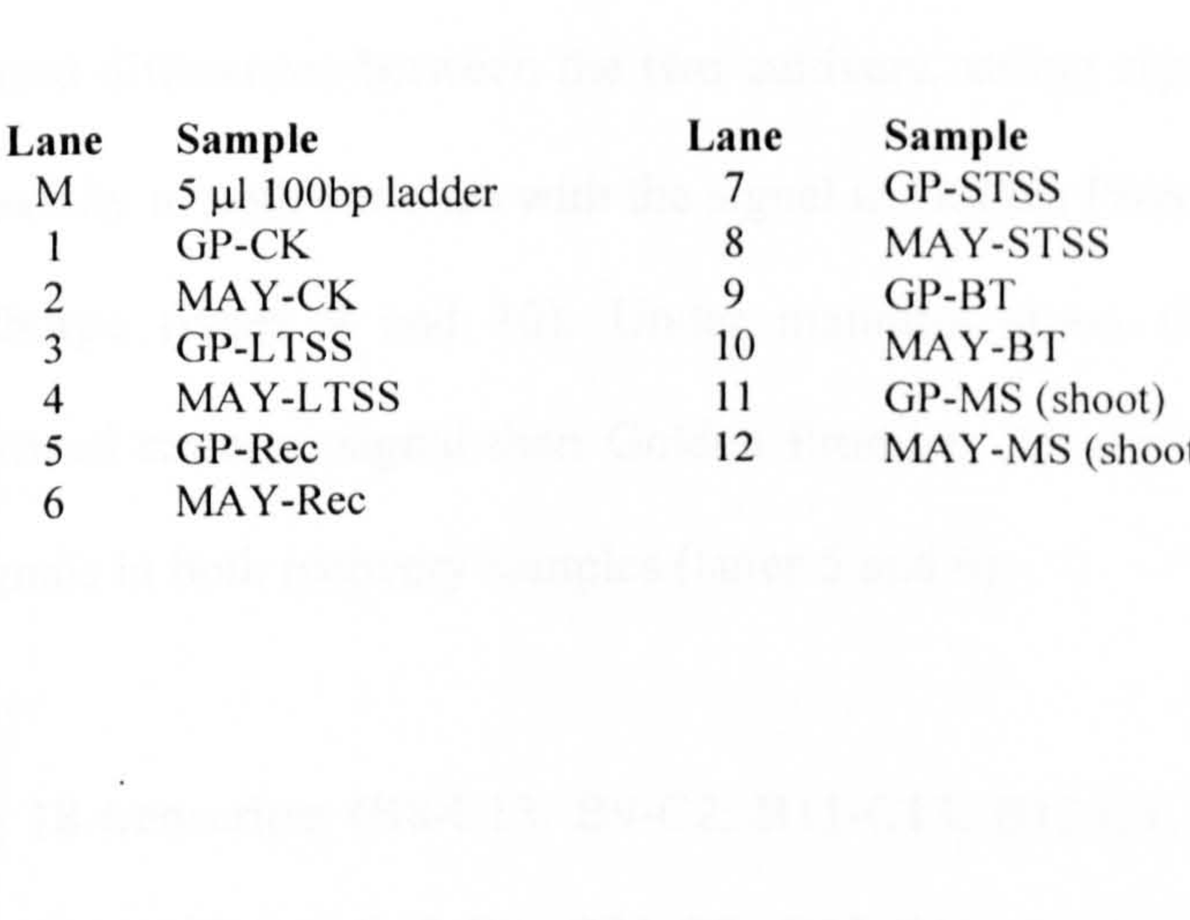
Fig. 3.14 1 % agarose gel of RT-PCR products of barley leaves of Golden Promise and Maythorpe with the primer combination PT4/R4, all lanes were loaded 15 µl of sample except for lane 9, which was loaded 7 µl.



Lane	Sample	Lane	Sample
M	5 µl 100bp ladder	7	MAY-STSS
1	MAY-CK	8*	GP-STSS
2	GP-CK	9*	GP-STSS (7 µl)
3	MAY-LTSS	10	GP-BT
4	GP-LTSS	11	MAY-BT
5	MAY-Rec	12	GP-MS (shoot)
6	GP-Rec	13	MAY-MS (shoot)

*Lane 8: the sample was left at room temperature overnight, in case DNA degradation, 7 µl of the previous PCR products was used in lane 9

Fig. 3.15 1 % agarose gel of RT-PCR products of barley leaves of Golden Promise and Maythorpe with the primer combination PT6/R5, all lanes were loaded 20 µl of sample.



Lane	Sample	Lane	Sample
M	5 µl 100bp ladder	7	GP-STSS
1	GP-CK	8	MAY-STSS
2	MAY-CK	9	GP-BT
3	GP-LTSS	10	MAY-BT
4	MAY-LTSS	11	GP-MS (shoot)
5	GP-Rec	12	MAY-MS (shoot)
6	MAY-Rec		

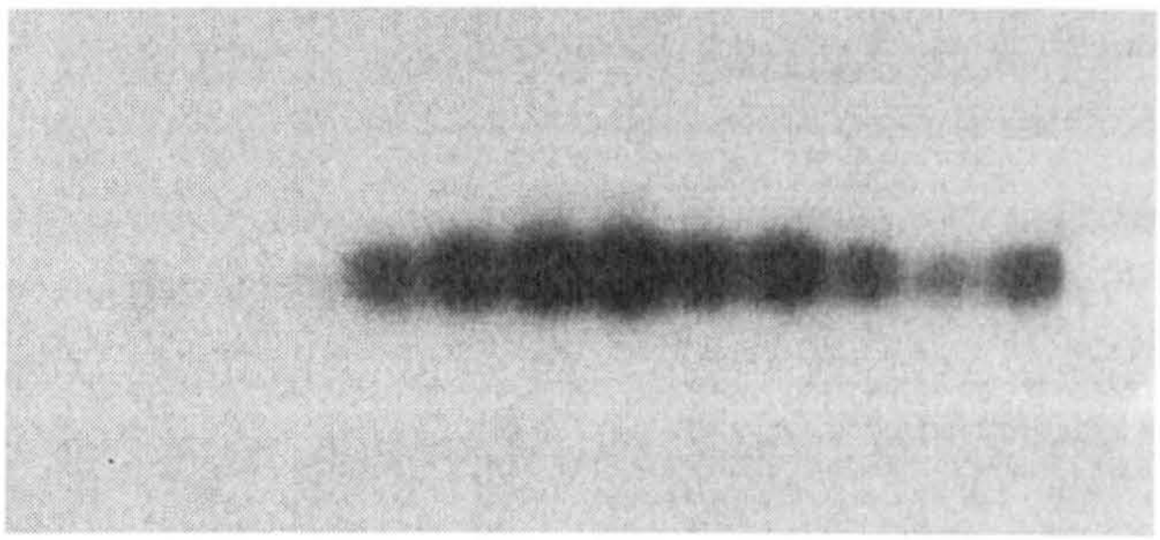
The blotted RT-PCR products of Golden Promise and Maythorpe were hybridised individually with the probes shown in Table 3.21. The results confirmed the positive clones of the differential bands. The clones could be grouped into three clusters based on their common induction patterns. B1-C4, B2-C19, B3-C4 and B4-C5 possessed similar Southern (slot) blot results (Fig. 3.16), they showed very weak signals in both controls of Golden Promise and Maythorpe (lanes 1 and 2) while GP-CK was slightly stronger than MAY-CK. Under long-term salt stress, Maythorpe possessed a stronger signal (lane 4) than Golden Promise (lane 3), with the latter showing no obvious difference when compared to its control (lane 1). Under short-term salt stress, both Golden Promise and Maythorpe showed strong positive signals while Golden Promise (lane 7) showed stronger signal than Maythorpe (lane 8). Ion toxicity and osmotic stress also caused differences between the two cultivars, strong signals were detected under boron toxicity in both varieties with the signal in Golden Promise being stronger than in Maythorpe (lanes 9 and 10). Under mannitol stress (lanes 11 and 12) Maythorpe showed stronger signal than Golden Promise. All the fragments showed very strong signals in both recovery samples (lanes 5 and 6).

The following 18 transcripts (B8-C13, B9-C2, B11-C13, B12-C1, B13-C4, B14-C3, B16-C2, B17-C12, B18-C3, B19-C9, B21-C2, B22-C11, B5-C5, B23-C1, B24-C4 B26-C5, B27-C4 and B28-C1) could be grouped together in one cluster as they had similar Southern (slot) blot results. One example hybridisation of B16-C2 is shown in Fig. 3.17. All these differential fragments had weak signals under control conditions with no difference between the two cultivars (lanes 1 and 2) except B22-C11 and B26-C5 where Maythorpe maintained a slightly stronger signal than Golden Promise. The major differences between the two cultivars were that more copies of the above

transcripts were amplified in Maythorpe both under short-term salt stress (lane 8) and long-term salt stress (lane 4) than in Golden Promise (lanes 7 and 3), with the latter maintaining very low signals in both these treatments. Maythorpe also maintained stronger signals (lane 12) than Golden Promise (lane 11) under osmotic stress. However, Golden Promise showed much stronger hybridisation than Maythorpe under recovery conditions (lanes 5 and 6) except B5-C5 and B23-C1 where no differences were detected between the two cultivars. There were no differences between Golden Promise and Maythorpe in response to ion toxicity with the exception of B5-C5, B27-C4, B16-C2, B22-C11, B23-C1, B24-C4 and B26-C5 where Golden Promise maintained stronger signals than Maythorpe (lanes 9 and 10).

Differential bands B15-C8, B20-C3 and B25-C1 produced similar Southern (slot) blot results. The hybridisation of B15-C8 is shown as an example of this cluster (Fig. 3.18). Golden Promise produced a stronger signal (lane 1) than Maythorpe (lane 2) under control conditions. Maythorpe, however, had more amplified fragments under short-term salt stress (lane 8), long-term salt stress (lane 4) and osmotic stress (lane 12) than Golden Promise (lanes 7, 3 and 11) respectively. In the recovery and ion toxicity treatments, Golden Promise (lanes 5 and 9) showed stronger signals than Maythorpe (lanes 6 and 10) except B25-C1, which produced a stronger signal in the Maythorpe recovery than in the Golden Promise recovery treatments.

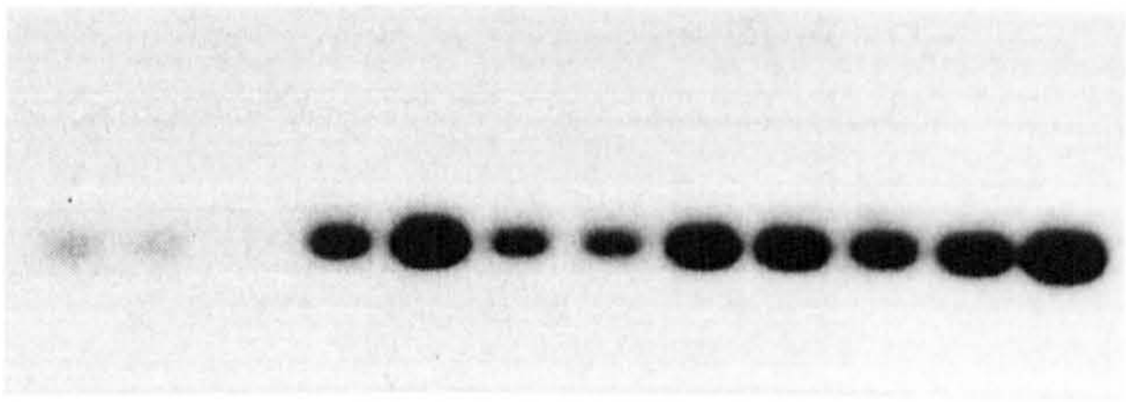
Fig. 3.16 Southern (slot) blot hybridisation results of B1-C4.



1 2 3 4 5 6 7 8 9 10 11 12

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Sample	GP-CK	MAY-CK	GP-LTSS	MAY-LTSS	GP-Rec	MAY-Rec	GP-STSS	MAY-STSS	GP-BT	MAY-BT	GP-MS	MAY-MS

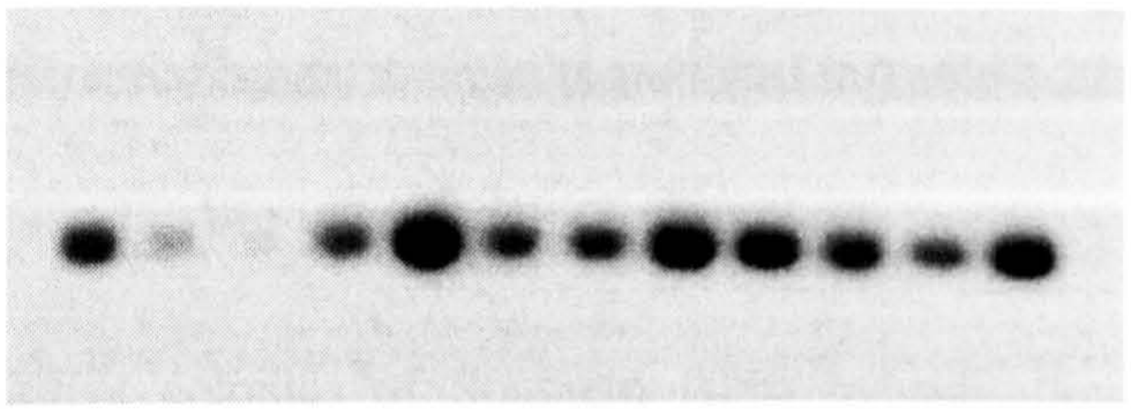
Fig. 3.17 Southern (slot) blot analysis result of B16-C2.



1 2 3 4 5 6 7 8 9 10 11 12

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Sample	GP-CK	MAY-CK	GP-LTSS	MAY-LTSS	GP-Rec	MAY-Rec	GP-STSS	MAY-STSS	GP-BT	MAY-BT	GP-MS	MAY-MS

Fig. 3.18 Southern (slot) blot analysis result of B15-C8.



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Sample	GP-CK	MAY-CK	GP-LTSS	MAY-LTSS	GP-Rec	MAY-Rec	GP-STSS	MAY-STSS	GP-BT	MAY-BT	GP-MS	MAY-MS

In summary, Southern (slot) blot analysis confirmed that all the differential bands had a positive signal plasmid DNA, so these plasmid DNA were chosen to represent their corresponding differential bands, the results are listed in Table 3.22.

Table 3.22 Positive colonies of Southern (slot) hybridisation of corresponding differential bands.

Band	Colony	Band	Colony
B1	B1-C4	B17	B17-C12
B2	B2-C19	B18	B18-C3
B3	B3-C4	B19	B19-C9
B4	B4-C5	B20	B20-C3
B5	B5-C5	B21	B21-C2
B8	B8-C13	B22	B22-C11
B9	B9-C2	B23	B23-C1
B11	B11-C13	B24	B24-C4
B12	B12-C1	B25	B25-C1
B13	B13-C4	B26	B26-C5
B14	B14-C3	B27	B27-C4
B15	B15-C8	B28	B28-C1
B16	B16-C2		

3.3.6 Northern blot analysis

All Northern blot analysis reported in this section was carried out using leaf samples. Seven Plasmid DNA samples (B1-C4, B12-C1, B28-C1, B15-C8, B20-C3, B22-C11, B26-C5) were used as probes to carry out Northern blot analysis. The results of B1-C4 showed that the size of RNA transcript hybridised to B1-C4 is about 1.9 kb in size (Fig. 3.19). This gene transcript was over-expressed in Maythorpe control leaves (lane

2) compared to Golden Promise (lane 1). STSS induced the expression of this gene in Golden Promise (lane 5) and seriously reduced the expression in Maythorpe (lane 6). LTSS did not change the expression of this gene in Golden Promise (lane 3) compared to the control (GP-CK), however, the expression was reduced in Maythorpe (lane 4) compared to its control (MAY-CK).

The expression of fragment B12-C1 was also differentially affected by salt stress between Golden Promise and Maythorpe (Fig. 3.20). Three RNA transcripts (3.5 kb, 2.0 kb and 1.4 kb) were hybridised to this fragment. The expression of transcripts of size 3.5 kb and 1.4 kb was slightly increased in Golden Promise under salt stress compared to its control, but slightly reduced by salt in Maythorpe. The major differential transcript is the middle size of about 2.0 kb and was over-expressed in Maythorpe control leaves (lane 2) compared to Golden Promise control (lane 1), which had very low expression. Under short-term salt stress, the expression of this gene transcript was up-regulated in Golden Promise (lane 5) and down-regulated in Maythorpe (lane 6) compared to their respective controls. LTSS also enhanced expression of this transcript in Golden Promise leaves (lane 3), but caused a reduction in expression in Maythorpe (lane 4) when compared to the respective controls.

In control treatments band B15-C8 (Fig. 3.21) was over-expressed in the leaves of Maythorpe (lane 2) compared to Golden Promise (lane 1). Both short-term and long-term salt stress caused up-regulation of the gene expression in Golden Promise (lanes 3 and 5), but down-regulation in Maythorpe (lanes 4 and 6). This RNA transcript is about 2.0 kb in size.

The RNA transcript hybridised by B20-C3 is about 1.9 kb in size, and was highly expressed under control conditions (Fig. 3.22) in Maythorpe (lane 2) when compared to Golden Promise (lane 1). STSS resulted in increased expression of this transcript in Golden Promise (lane 5) but a reduction in expression in Maythorpe (lane 6) compared to the respective controls. LTSS also caused a reduction in the expression of this gene transcript in Maythorpe (lane 4) related to the control, but this gene transcript was not over-expressed in Golden Promise under LTSS (lane 3).

Two different size RNA transcripts (Fig. 3.23) were hybridised to band B22-C11, their sizes are approximately 1.9 and 1.4 kb, respectively. The expression patterns of these transcripts were quite similar under salt stress, in that both were over-expressed in Maythorpe (lane 2) compared to Golden Promise (lane 1). STSS and LTSS caused a reduction in expression of this transcript in Maythorpe (lanes 4 and 6) related to the control. The response of Golden Promise to salt stress was different, in that STSS induced expression of these transcripts (lane 5) but LTSS resulted in no obvious difference in expression (lane 3) when compared to the control treatment (lane 1). Although the two different size RNA transcripts showed similar expression patterns in relation to salinity stress, the expression of the transcript of 1.4 kb showed obvious differences between Golden Promise and Maythorpe compared to the transcript of 1.9 kb.

The Northern blot analysis results of B26-C5 are shown in (Fig. 3.24), this fragment hybridised to six different size RNA transcripts, which were about 3.5, 2.6, 1.5, 1.3, 1.0 and 0.8 kb, respectively. All these transcripts were over-expressed in Maythrope (lane 2) compared to Golden Promise (lane 1) with the exception of the smallest

transcript of 0.8 kb, which showed no difference in expression between the two cultivars under control conditions. In Maythorpe all transcripts were down-regulated in response to STSS (lane 6) and LTSS (lane 4). However, no differences in expression of the 3.5, 2.6, 1.3 and 0.8 kb transcripts were observed between the STSS and LTSS, but the 1.5 and 1.0 kb transcripts showed increased expression under LTSS than under STSS. In Golden Promise, the 1.0 kb transcript was up-regulated under LTSS (lane 3) and the 0.8 kb transcript was slightly down-regulated by STSS (lane 5) and LTSS (lane 3) when compared to the control treatment (lane 1). Expression of the remaining transcripts was not affected by salinity either STSS or LTSS in Golden Promise. All of the six RNA transcripts showed very low expression under salt stress with just the 1.5 and 1.0 kb transcripts showing varied differences in expression. A comparison between the two cultivars in response to salinity showed that the 1.5 kb transcript was slightly over-expressed in Maythorpe (lane 4) compared to Golden Promise (lane 3) while the 1.0 kb transcript was slightly over-expressed in Golden Promise (lane 3) compared to Maythorpe (lane 4) under LTSS. No differences between cultivars were observed for remaining transcripts under both STSS and LTSS.

Northern blot analysis with the B28-C1 probe could not produce any signals (data not shown).

Fig. 3.19 Northern hybridisation of B1-C4. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.

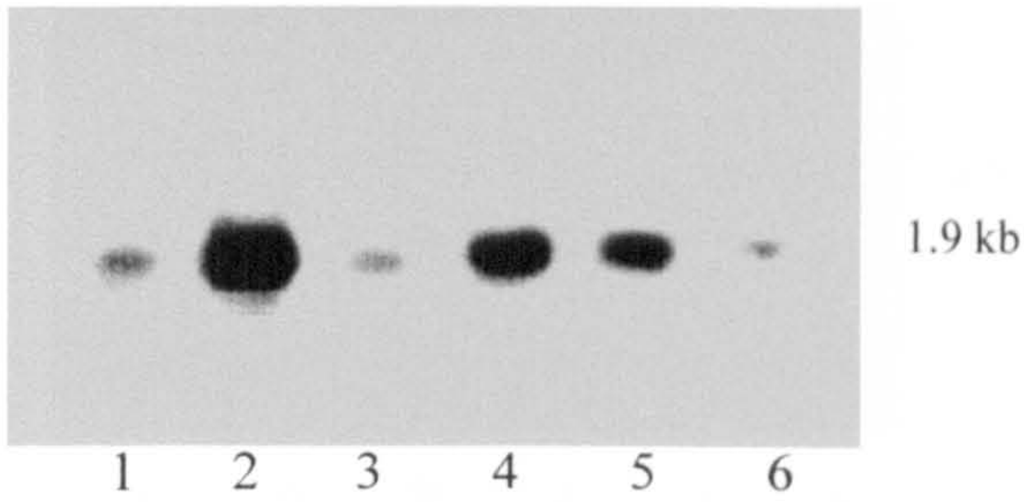


Fig. 3.20 Northern hybridisation of B12-C1. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.

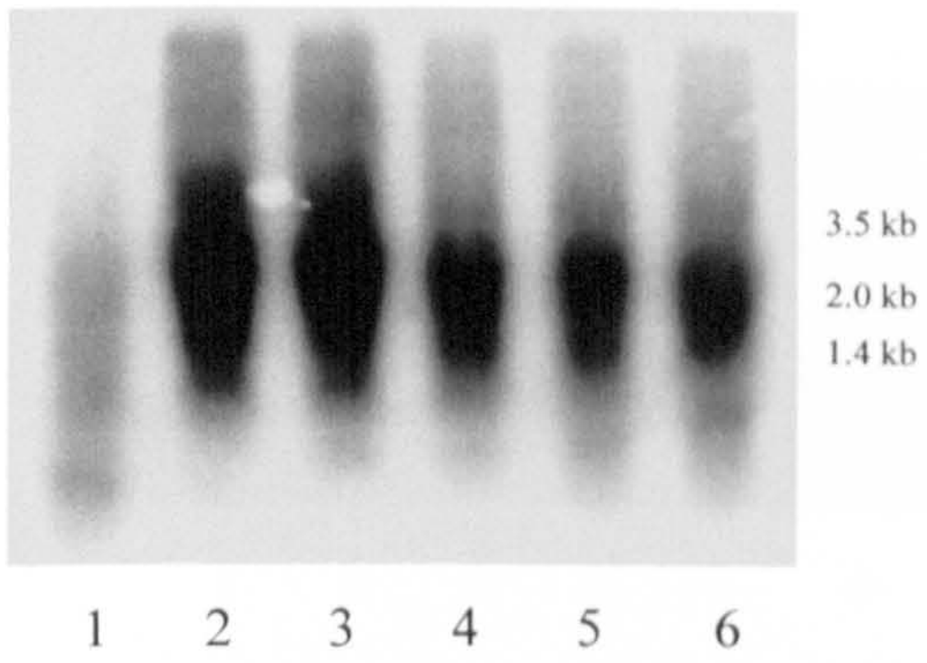


Fig. 3.21 Northern hybridisation of B15-C8. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.

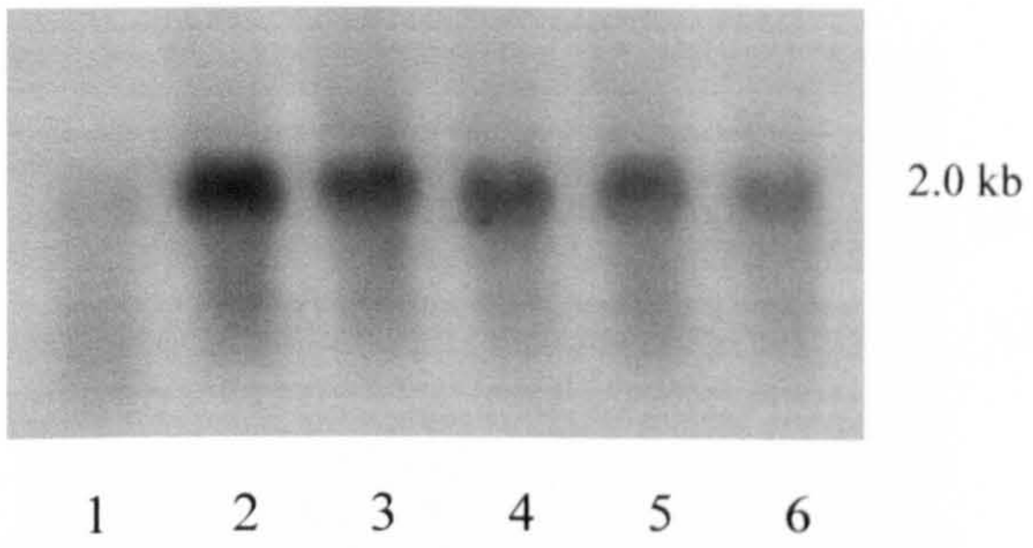


Fig. 3.22 Northern hybridisation of B20-C3. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.

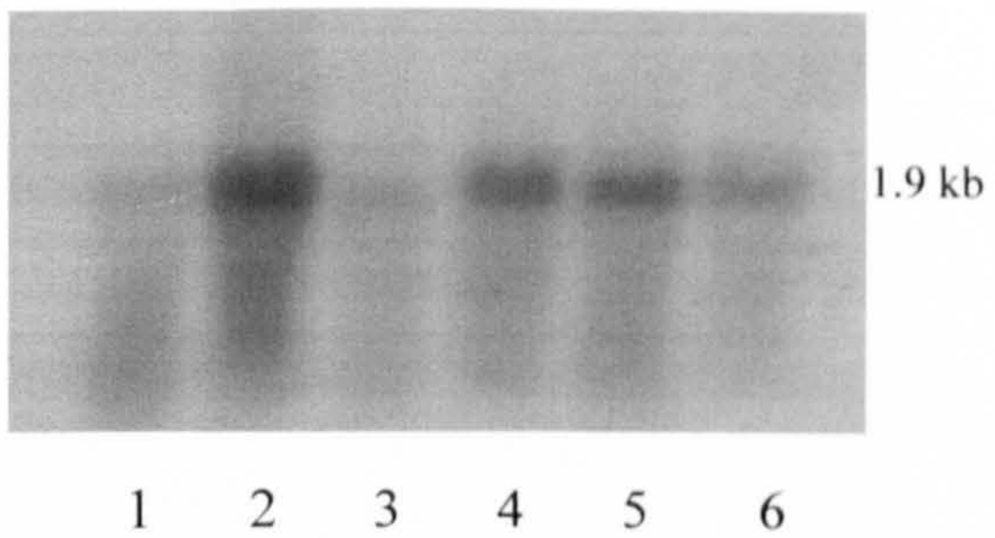


Fig. 3.23 Northern hybridisation of B22-C11. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.

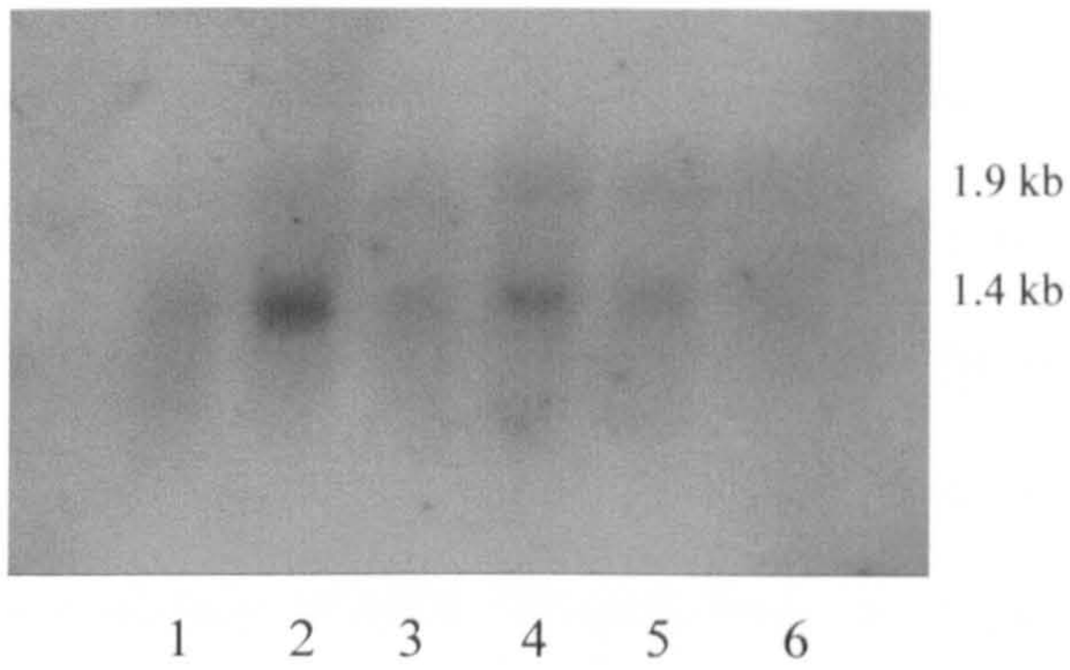
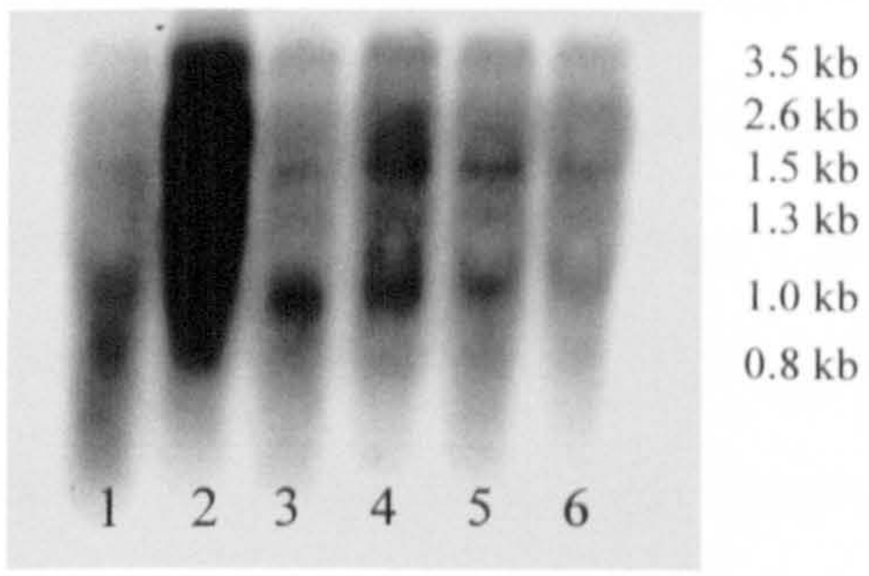


Fig. 3.24 Northern hybridisation of B26-C5. Lane 1 to 6 are GP-CK, MAY-CK, GP-LTSS, MAY-LTSS, GP-STSS and MAY-STSS, respectively.



3.4 Discussion

The differential display technique was successfully used in that a number of *H. vulgare* transcripts were identified that showed different patterns of expression between the cultivars Golden Promise and Maythorpe. Initial experiments were unsuccessful because of problems with the preliminary RT step rather than the latter PCR. Both one-base anchored oligo-dT primers and two-base anchored oligo-dT primers consist of 11 and 12 Ts, respectively. They were used in the RT reactions and the consequent PCR of barley samples. One-base anchored oligo-dT primers did not work well although similar primers were introduced by Liang *et al.* (1994) and successfully used in differential display of HEK293 cells (Von der Kammer *et al.*, 1999). The reason for failure in RT-PCR of barley samples in this work may be that one base anchored oligo-dT primers have less chance to anneal poly-A tail and adjacent region, most of this primer might only anneal within poly-A tail. Besides poly-T, two-base anchored oligo-dT primers consist of two additional 3' bases, which provide more specificity than one base to anchore to the two complementary bases located just upstream of the poly-A tails of mRNAs (Liang and Pardee, 1992). With two-base anchored oligo-dT primers, a vast number of amplified cDNA fragments from leaf tissue were similar between the two cultivars as would be expected from the close genetic relationship between the two cultivars. Only 28 differential bands were identified between Golden Promise and Maythorpe, these bands were a very small portion (about 2 percent) of those amplified fragments. This means the differential bands could represent key changes in gene expression between the two cultivars and in response to stress. The differential expressions of the transcripts between the two cultivars under control and stress conditions could be related to the *ari-e*.GP mutation (Pakniyat *et al.*, 1997a). Among the 28 differential bands, 8 bands (B27, B15, B16,

B17, B18, B19, B20 and B22) were identified as varietal differential bands under control conditions, 8 bands (B3, B8, B9, B28, B21, B23, B24 and B25) were differential bands induced by salt stress, 5 bands (B1, B2, B11, B13 and B14) were caused by general stress including salt, ion toxicity and osmotic stresses, and 7 bands (B4, B5, B6, B7, B10, B12 and B26) were induced by ion toxicity and osmotic stress. It is essential to carry out further confirmation after differential bands were isolated from the polyacrylamide gels because the differential bands excised from them may also contain false signals due to PCR amplification and co-migration of similar size fragments (Von der Kammer *et al.*, 1999). In the present experiment, for example, several differential bands had two fragments, *e.g.* B11, B12 and B23 after the cDNA fragments were eluted and amplified by PCR. In addition, most of the bands possessed different size inserts detected by restriction digestion of plasmid DNAs. Restriction digestion with a 6 base cutter enzyme such as *Eco*R I is a very useful, efficient and cheap technique to screen different length inserts. One reason is pGEM-T Easy vector has two *Eco*R I cutting sites at the both ends of the insert cDNA fragment. In theory a 6 base cutter enzyme will cut once every 4096 bp, most of the differential fragments cut from polyacrylamide gels were about 500 bp long, so it is unlikely that a relatively short cDNA will contain a cutting site. The cut fragment from plasmid DNA can represent the inserted transcript. Screening results showed varied size fragments in most of the differential bands, which indicated that both true and false signals may exist. To minimise the false signals, Southern (slot) blot analysis was a useful and quick technique. Because RT-PCR products were used as DNA source and blotted onto a nylon membrane, it was easy to pick out the positive differential signals using probes from cloned cDNA. For example, two different size cDNA inserts (B3-C1 and B3-C4) of band B3 were detected by restriction digestion screening, the blot results

showed that B3-C4 was a positive differential fragment but no signals were produced by B3-C1. B15 is another example, in that its clone B15-C8 showed positive and differential signals between Golden Promise and Maythorpe but the other clone B15-C6 with different size insert produced no hybridisation signal. With this technique the positive differential fragments of all the bands were confirmed.

Northern blot analysis used cloned differential cDNA probes to directly hybridise mRNA transcripts. This technique is a key method used to investigate gene expression of corresponding differential cDNA fragments. Northern blot analysis would allow a semi-quantitative assessment of gene expression and provide the approximate size of mRNA transcripts based on the signals and distance of migration.

Differences between the results of Southern (slot) blot and Northern blot analysis were observed in this experiment. For instance, Southern (slot) blot analysis showed that B1-C4 produced very weak signals in both GP-CK and MAY-CK although the signal of GP-CK was slightly stronger than MAY-CK, Northern blot results indicated MAY-CK over-expressed the gene transcript hybridised to B1-C4 compared to GP-CK, while the same observations for B1-C4 were detected by the both techniques under short-term and long-term salt stress. B15 is another example, this band was strongly expressed in GP-CK from the differential display gel and was confirmed by Southern (slot) blot analysis. However, Northern blot analysis indicated that the mRNA tanscript hybridised to B15-C8 was over-expressed in MAY-CK rather than GP-CK. These differences between Southern (slot) blot and Northern blot analysis indicated that the signals from the former technique (obtained by probing RT-PCR products) could not always be used to accurately explain gene expression. Because RT-PCR

products are partial sequences of mRNA transcripts, whether a mRNA transcript can be reverse transcribed to a cDNA sequence with a specific two-base anchored oligo-dT primer is largely dependent on the nucleotides connected to the poly-A tail. If Golden Promise and Maythorpe produced similar amount of the same mRNA transcript, the consequent RT-PCR with a designated primer combination would produce very similar results without differential fragments. If these two plants expressed different levels of mRNA transcript, then RT-PCR with a designated primer combination would show differential bands between the two cultivars. If the two plant cultivars expressed similar mRNA transcripts but with different poly-A tails, RT and the following PCR with the oligo-dT primer may show expression in one cultivar but not in the other and in this case the differential bands may not reflect the differences in gene expression.

Additionally, PCR might be another cause of these discrepancies. Several factors can affect the yield of cDNA, *e.g.* the initial amount of DNA template, the annealing temperature, the magnesium concentration and running cycles. A "plateau effect" of PCR could result in a non-proportional amplification of the template DNA fragments (McPherson and Møller, 2000). Because when the amplification of a target sequence reaches a certain level, the products could form dimmers, which would result in slowing down the amplification of the product like "plateau". Meanwhile, the primers would anneal to other transcripts, even non-specific fragments, as a result, these amplification would be speed up. Another important factor is primer and primer combination, some primer combinations such as PT6/R5 and PT4/R3 worked more efficiently than others such as PT5/R4 and PT4/R1 in this study.

In conclusion, mRNA differential display is an efficient technique to be used to identify differentially expressed genes although some false cDNA fragments can be produced during the procedures, which can be minimised by using cloning and Southern (slot) blot analysis. Southern (slot) blot analysis is a useful technique to confirm positive differential fragments but Northern hybridisation is the key technique to show patterns in gene expression. The 28 differential cDNA fragments were cloned from Golden Promise and Maythorpe in relation to salt and other stresses. To further understand possible functions of related genes, the fragments were sequenced and submitted to GenBank to perform DNA sequence analysis by comparison with known gene sequences in the database.

Chapter 4

cDNA sequence and homology analysis

4.1 Introduction

Classically genes were identified as units of function by the isolation and characterisation of their corresponding mutant phenotypes. Molecular cloning techniques greatly speed this process by eliminating the necessity for the study of precise mutations and their phenotypes. The method relies upon similarity at the DNA sequence level with known genes or by related comparisons of their putative protein products. The cDNA fragments isolated with the differential display technique are normally about 500 bp or less in size due to using decamer random primers and oligo-dT anchored primers (Liang *et al.*, 1992). These fragments are normally partial sequences from mRNA transcripts.

To understand the functions of these short sequences, the cDNA and deduced amino-acid sequences were analysed by sequence comparison to the GenBank database using computer program BLAST 2.0 (the Basic Local Alignment Search Tool) with default parameters (Altschul *et al.*, 1997). This approach has been widely used as a method that can efficiently supply information relating to corresponding genes, such as name, identity and function based on similarities with previously characterised genes and proteins. Examples of the success of using the BLAST facility are provided by the barley peroxisomal ascorbate peroxidase gene (*HvAPXI*) identified from salt-stressed leaves (Shi *et al.*, 2001), and an α-subunit of the mitochondrial processing peptidase (α-MPP) expressed in UV-stressed leaves of mulberry (Sharma *et al.*, 2001). Both

genes were identified using the GenBank database following differential display. Several databases in GenBank are available for comparison analysis with barley EST using BLAST searching facilities, such as BLASTN which queries a nucleotide sequence against an EST database (dbEST) and a non-redundant (nr) nucleotide database. BLASTX (http://www.ncbi.nlm.nih.gov) translates a nucleotide query into all six frames and searches a protein database (Benson *et al.*, 2002). The output of BLAST search results is listed with an E-value (Expectation value: The probability of an unrelated or a random sequence in the database achieving a similar or better score with the query sequence. A low value for a retrieved sequence signifies higher similarity, Peri *et al.*, 2001). The portion of sequences could not be well characterised if the E-value exponent is less than –5 (Waugh *et al.*, 2000). Other databases can also be used to search homologues of barley EST's, such as 'UK cropNet' (http://www.ukcrop.net, Dicks *et al.*, 2002) and 'The Arabidopsis Information Resource' (TAIR) (http://www.arabidopsis.org/home.html, Huala *et al.*, 2001). However, this comparison is very much dependent on the capacity of the present GenBank database.

Some EST fragments may not be significantly homologous to any genes because the corresponding genes have not as yet been identified (Liang *et al.*, 1993, Von der Kammer *et al.*, 1999). As the database of GenBank is increasing and with the completion of sequencing of the whole genome of some plants such as *A. thaliana* (http://www.arabidopsis.org), more and more genes will be identified. Homology analysis will in the future become more powerful in understanding gene expression and function. In this experiment, all of the cloned differential EST sequences (B1-B28, Chapter 3) were submitted to GenBank to search for homologous genes using the

BLAST facility through dbEST, DNA and protein databases. The objective of this Chapter is to assign possible functions to the differential display products isolated in Chapter 3 using homology comparisons on databases.

4.2 Materials and methods

4.2.1 Sequencing cDNA

The colonies from pGEM-T clones confirmed by Southern (slot) blot analysis were cultured in LBA overnight following the method in 3.2.8. Plasmid DNA purification was carried out with the Wizard Plus Midipreps DNA purification System (Promega, Madison, WI, USA). Briefly, 100 ml of cultured cells was centrifuged at 4,000 ×g for 10 min (4 °C), the supernatant was discarded and the tube was blotted upside down on a paper towel to remove excess liquid. The pellet was completely resuspended in 3 ml of cell resuspension solution (Appendix 22) and 3 ml of cell lysis solution (Appendix 23) was added, mixing occurred by inverting the tube four times followed by the addition of 3 ml of neutralisation solution (Appendix 24) prior to inverting the tube 4 times. The tube was then centrifuged at 12,000 ×g for 15 min (4 °C). The supernatant was carefully transferred to a new centrifuge tube and 10 ml of resuspended Wizard Midipreps DNA purification resin was added. A Midicolumn tip was meanwhile inserted into the vacuum manifold port. After transferring the resin/DNA mixture into the Midicolumn, a vacuum was applied. When all of the sample had passed through the column, 15 ml of column wash solution (Appendix 25) was added to the Midicolumn. This procedure was repeated twice. The resin was dried by continuing to draw a vacuum for 30 seconds after the solution was pulled through the column. The Midicolumn was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12,000 ×g for 2 min. The Midicolumn was then transferred to a new microcentrifuge

tube followed by the addition of 200 µl of preheated (65 °C - 70 °C) nuclease free water. After 1 min, the plasmid DNA was eluted by centrifugation at 12,000 ×g for 5 min and stored at –20 °C.

The concentration and purity of the purified plasmid DNA were estimated by restriction digestion (Table 4.1) and electrophoresis on a 1 % agarose gel. About 30 µg of the purified plasmid DNA was sent to MWG-Biotech (Ebersberg, Germany) for sequencing, using an automated DNA Sequencer.

Table 4.1 Protocol for restriction digestion of plasmid DNA.

Component	Volume (µl)
dH_2O	15
10×buffer H	2
Plasmid DNA	2
Pst I	1
Incubation overnight at 37 °C	

4.2.2 cDNA sequence analysis

Analysis of the cDNA sequences was carried out by sequence comparison to the nr GenBank database including EST and nr nucleotide sequence using the Basic Local Alignment Search Tool (BLAST 2.0) with default parameters (Altschul *et al.*, 1997).

Putative peptide sequences of the differential bands were deduced from their corresponding nucleic acid sequences using the universal genetic code. The analysis of

deduced amino-acid sequences was performed by comparison to the nr peptide sequence database of GenBank using BLASTX software with default parameters (Altschul *et al.*, 1997).

4.3 Results

All of the sequences isolated in this study were registered in the dbEST of GenBank and their GenBank accession numbers are presented directly following the corresponding differential band number.

4.3.1 Homology analysis of B1/B4, B2, B3 and B5/B23

The six cDNA fragments B1/B4 (GenBank accession number: BF704574), B2 (GenBank accession number: BF718804), B3 (GenBank accession number: BF718805) and B5/B23 (GenBank accession number: BF718806) were found to have similar sequences (Fig 4.1). Bands B1 and B4 had the same sequence, which was expressed as B1/B4, while B5 and B23 also had the same sequence, which was expressed as B5/B23. These sequences show that the R3 random primer sequence was located at the 5' end and the antisense oligo-dT PT4 primer sequence was at the 3' end with the exception of band B2, which had the R3 antisense sequence instead. The sequence of B1/B4 had 321 bases, which was the longest among the above four transcripts. The following analysis of the other three sequences is based on the sequence of B1/B4. The sequence of B5/B23 was 289 bases long and was 100 % identical to B1/B4 with the exception of 32 bases short at the 3' end. The putative protein sequences of both these sequences are also the same. The sequence of B2 had just 217 bases and did not contain a poly-A tail due to the fact that the downstream primer was the antisense R3 random primer. The first 183 bases of B2 were identical to B1/B4

with the exception of two different nucleotides at positions 43 and 120 bases, where T and C were replaced by C and T, respectively. They are highlighted in Fig. 4.1. The first replacement caused a different amino-acid where histidine (H) replaced tyrosine (Y). In addition to this, 34 bases from the 3' end of B2 were very different from B1/B4 but they were out of the reading frame. The sequence of band B3 was 259 bases long, the major difference of this sequence to that of B1/B4 was that two thymines (T) were inserted at base positions 54 and 131 (highlighted by the black background in Fig. 4.1). These insertions caused changes in the reading frame, with the putative peptide sequence being different from B1/B4 from the first T insertion. This part of the peptide sequence contained 8 leucines within 55 amino-acids.

Fig. 4.1 cDNA and putative protein sequences of B1/B4, B2, B3 and B5/B23. The similarities in sequences are highlighted in light grey background and primer sequences are underlined. The putative protein sequences were produced based on the reading frame of the wheat ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (X00235).

```
B1/B4   1    TACAACGAGGTGGAGGAGGTCAAGAAGGAGTACCCTGACGCGTATGTC   48
                E  V  E  E  V  K  K  E  Y  P  D  A  Y  V
B2      1    TACAACGAGGTGGAGGAGGTCAAGAAGGAGTACCCTGACGCGCATGTC   48
                E  V  E  E  V  K  K  E  Y  P  D  A  H  V
B3      1    TACAACGAGGTGGAGGAGGTCAAGAAGGAGTACCCTGACGCGTATGTC   48
                E  V  E  E  V  K  K  E  Y  P  D  A  Y  V
B5/B23  1    TACAACGAGGTGGAGGAGGTCAAGAAGGAGTACCCTGACGCGTATGTC   48
                E  V  E  E  V  K  K  E  Y  P  D  A  Y  V

B1/B4   49   CGCATCATCGGATTCGACAACATGC GTCAGGTGCAGTGCGTCAGCTTC   96
              R  I  I  G  F  D  N  M   R  Q  V  Q  C  V  S  F
B2      49   CGCATCATCGGATTCGACAACATGC GTCAGGTGCAGTGCGTCAGCTTC   96
              R  I  I  G  F  D  N  M   R  Q  V  Q  C  V  S  F
B3      49   CGCATCATCGGATTCGACAACATGCTGTCAGGTGCAGTGCGTCAGCTTC   97
              R  I  I  G  F  D  N  M  L  S  G  A  V  R  Q  L
B5/B23  49   CGCATCATCGGATTCGACAACATGC GTCAGGTGCAGTGCGTCAGCTTC   96
              R  I  I  G  F  D  N  M   R  Q  V  Q  C  V  S  F

B1/B4   97   ATCGCCTTCAAGCCACCAGGCTGCGAGGAGTCC GGCAAGGCATAAACA  144
              I  A  F  K  P  P  G  C  E  E  S   G  K  A
B2      97   ATCGCCTTCAAGCCACCAGGCTGTGAGGAGTCC GGCAAGGCATAAACA  144
              I  A  F  K  P  P  G  C  E  E  S   G  K  A
B3      98   ATCGCCTTCAAGCCACCAGGCTGCGAGGAGTCCTGGCAAGGCATAAACA  146
              H  R  L  Q  A  T  R  L  R  G  V  L  A  R  H  K  Q
B5/B23  97   ATCGCCTTCAAGCCACCAGGCTGCGAGGAGTCC GGCAAGGCATAAACA  144
              I  A  F  K  P  P  G  C  E  E  S   G  K  A

B1/B4   145  GCTCACTGACGATGGGCCACATATAAAGTGCCATTGCAGTTTTGTCAA   192
B2      145  GCTCACTGACGATGGGCCACATATAAAGTGCCATTGCAGGGTACTCCT   192
B3      147  GCTCACTGACGATGGGCCACATATAAAGTGCCATTGCAGTTTTGTCAA   194
               L  T  D  D  G  P  H  I  K  C  H  C  S  F  V  N
B5/B23  145  GCTCACTGACGATGGGCCACATATAAAGTGCCATTGCAGTTTTGTCAA   192

B1/B4   193  CTCTGACATTGCTTTGGGTTTTCCTTCTCCATTTATCTTTCTTATTTG   240
B2      193  TCTTGACCTCCTCCACCTCGTTGTA                         217
B3      195  CTCTGACATTGCTTTGGGTTTTCCTTCTCCATTTATCTTTCTTATTTG   242
              S  D  I  A  L  G  F  P  S  P  F  I  F  L  I  C
B5/B23  193  CTCTGACATTGCTTTGGGTTTTCCTTCTCCATTTATCTTTCTTATTTG   240

B1/B4   241  TTCCTAAGAATATGTGTATGTCCATGTTCATGTACCAACATGGCTCGA   288
B3      243  TTCCGAAAAAAAAAAAA                                 259
B5/B23  241  TTCCTAAGAATATGTGTATGTCCATGTTCATGTACCGAAAAAAAAAAAA   289

B1/B4   271  GAAAACATGCTTGTATGCGAAAAAAAAAAAAA                   321
```

By searching in the dbEST database of GenBank with BLASTN, bands B1/B4, B3 and B5/B23 were 100 % (303/303 bp), 99 % (242/244 bp) and 100 % (274/274 bp) homologous to *Blumeria* infected *H. vulgare* EST clones BE214567, BE214079 and BF266779, respectively. Band B2 showed a high homology (180/182 bp, 98 %) to the *Blumeria* infected *H. vulgare* EST clones BI953316, BF265616 and BF264997 (Table 4.2).

Table 4.2 The top three homologous EST results of differential fragments B1/B4, B2, B3 and B5/B23 from the dbEST of GenBank.

	B1/B4	B2	B3	B5/B23
1	BE214567 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 303/303 bp (100 %)	BI953316 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 180/182 bp (98 %)	BE214567 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 242/244 bp (99 %)	FE214567 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 274/274 bp (100 %)
2	BE214079 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 303/303 bp (100 %)	BF265616 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 180/182 bp (98 %)	BE214079 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 242/244 bp (99 %)	BE214079 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 274/274 bp (100 %)
3	BF266779 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 303/303 bp (100 %)	BF264997 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 180/182 bp (98 %)	BF266779 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 242/244 bp (99 %)	BF266779 *H. vulgare* seedling green leaf cDNA (*Blumeria* infected) 274/274 bp (100 %)

The top three homologues of bands B1/B4, B2, B3 and B5/B23 from the nr nucleotide sequence database of GenBank are shown in Table 4.3. All four of these transcripts were significantly and constantly homologous to the gene *rbcS* encoding ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit of cereal plant species, barley (*H. vulgare*), wheat (*T. aestivum*) and *Aegilops squarrosa*. They were significantly homologous to the wheat mRNA for the small subunit of a precursor of Rubisco (X00235), the identities were 291/299 bp (97 %), 169/177 bp (95 %), 232/240 bp (96 %) and 264/270 bp (97 %), respectively. In addition, B2 and B3 showed high homology to the *H. vulgare* mRNA for Rubisco small subunit (U43493) while B1/B4 and B5/B23 showed high homology to *A. squarrosa* mRNA for Rubisco small subunit (X83095), the identities were 94 % or higher. Besides the top three homologues, the rest of the matches were all for the Rubisco small subunit including other plant species such as *Avena strigosa* and *Agropyron intermedium*. The results indicated that the differential bands B1/B4, B2, B3 and B5/B23 were the gene transcripts for Rubisco small subunit (*rbcS*).

Table 4.3 The top three genes homologous to B1/B4, B2, B3 and B5/B23 from the nr nucleotide sequence database of GenBank.

	B1/B4	B2	B3	B5/B23
1	X00235 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 291/299 bp (97 %)	U43493 *H. vulgare* Rubisco small subunit mRNA 174/181 bp (96 %)	X00235 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 232/240 bp (96 %)	X00235 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 264/270 bp (97 %)
2	X00234 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 289/303 bp (95 %)	X00235 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 3169/177 bp (95 %)	X00234 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 234/244 bp (95 %)	X00234 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 262/272 bp (96 %)
3	X83095 *A. squarrosa* mRNA for Rubisco small subunit 284/301 bp (94 %)	X00234 *T. aestivum* mRNA fragment for small subunit precursor of Rubisco 171/181 bp (94 %)	U43493 *H. vulgare* Rubisco small subunit mRNA 226/236 bp (94 %)	X83095 *A. squarrosa* mRNA for Rubisco small subunit 257/272 bp (94 %)

The homology analysis of the putative peptide sequences of B1/B4, B2, B3 and B5/B23 further confirmed that they are gene transcripts encoding the small subunit of Rubisco (Table 4.4). The translated sequences of the four cDNAs showed high similarity to the three wheat Rubisco small subunits (BAB19812, BAB19810 and CAA25057) although their identities varied depending on the length of the differential fragments. The identities of bands B1/B4 and B5/B23 were 45/45 amino-acids (100 %), band B2 was 44/45 amino-acids (99 %) and band B3 was 100 % to two separate peptides (23/23 and 18/18 amino-acids). The alignment figure for the cDNA and

putative protein sequences of the differential band B1/B4 to wheat Rubisco small subunit (X00235) are shown in Fig. 4.2. It shows that both DNA and protein sequences are significantly homologous, the differences of a few nucleotides in the encoding region of DNA sequences did not alter the peptide sequence, and most of the differences at the DNA level are outside the open reading frame in the untranslated region. Besides the top three homologoues, the rest of the matches were all to the Rubisco small subunit including other plant species such as barley.

Table 4.4 The top three genes from the nr peptide sequence database of GenBank homologous to B1/B4, B2, B3 and B5/B23.

	B1/B4	B2	B3	B5/B23
1	BAB19812 (AB042066) Wheat Rubisco small subunit 45/45 amino-acids, 100 %	BAB19812 (AB042066) Wheat Rubisco small subunit 44/45 amino-acids, 97 %	BAB19812 (AB042066) Wheat Rubisco small subunit 23/23 and 18/18 amino-acids, 100 %	BAB19812 (AB042066) Wheat Rubisco small subunit 45/45 amino-acids, 100 %
2	BAB19810 (AB042064) Wheat Rubisco small subunit 45/45 amino-acids, 100 %	BAB19810 (AB042064) Wheat Rubisco small subunit 44/45 amino-acids, 97 %	BAB19810 (AB042064) Wheat Rubisco small subunit 23/23 and 18/18 amino-acids, 100 %	BAB19810 (AB042064) Wheat Rubisco small subunit 45/45 amino-acids, 100 %
3	CAA25057 (X00234) Wheat Rubisco small subunit 45/45 amino-acids, 100 %	CAA25057 (X00234) Wheat Rubisco small subunit 44/45 amino-acids, 97 %	CAA25057 (X00234) Wheat Rubisco small subunit 23/23 and 18/18 amino-acids, 100 %	CAA25057 (X00234) Wheat Rubisco small subunit 45/45 amino-acids, 100 %

Fig. 4.2 Homology of the cDNA and the putative protein sequences of B1/B4 to the wheat ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (X00235); identical sequences are highlighted.

```
B1/B4:     7 gaggtggaggaggtcaagaaggagtaccctgacgcgtatg  46
               E  V  E  E  V  K  K  E  Y  P  D  A  Y
X00235:  359 gaggtggaggaggtcaagaaggagtaccctgacgcgtatg 398
               E  V  E  E  V  K  K  E  Y  P  D  A  Y

B1/B4:    47 tccgcatcatcggattcgacaacatgcgtcaggtgcagtg  86
               V  R  I  I  G  F  D  N  M  R  Q  V  Q  C
X00235:  399 tccgcatcatcggattcgacaacatgcgtcaggtgcagtg 438
               V  R  I  I  G  F  D  N  M  R  Q  V  Q  C

B1/B4:    87 cgtcagcttcatcgccttcaagccaccaggctgcgaggag 126
                 V  S  F  I  A  F  K  P  P  G  C  E  E
X00235:  439 cgtcagcttcatcgccttcaagccaccgggctgcgaggag 478
                 V  S  F  I  A  F  K  P  P  G  C  E  E

B1/B4:   127 tccggcaaggcataaacagctcactgacgatgggccacat 166
               S  G  K  A
X00235:  479 tccggcaaggcctaaacagctcactcacgacaggccatat 518
               S  G  K  A

B1/B4:   167 ataaagtgccattgcagttttgtcaactctgacattgctt 206
X00235:  519 ataaagtgccattgcagttttgtcaactctgacattgctt 558

B1/B4:   207 tgggttttccttctccatttatctttcttatttgttccta 246
X00235:  559 tgggttttccttctccatttatctttcttatttgttccta 598

B1/B4:   247 agaatatgtgtatgtccatgttcatgtaccaacatggctc 286
X00235:  599 agaatatgtgtatgtccatgttcatgtaccaacatggctc 638

B1/B4:   287 gagaaaacatgcttgtatg                      305
X00235:  639 gagaaagcatgctcgtatg                      657
```

4.3.2 Homology analysis for the sequences B15 and B20

The cDNA sequence of differential band B15 (GenBank accession number: BF718807) was 270 bases long and presented with the reverse sequence (B15R), B20 (GenBank accession number: BF718808) was 246 bases long (Fig. 4.3). Both transcripts had a random primer (R5) sequence at the 5' end and B20 showed a poly-A tail at the 3' end. Although no poly-A tail was attached to the sequence of B15R, the four nucleotide residue CTCA at the 3' end was very similar to poly-A adjacent region CTAA of B20. It is possible that B15 was also amplified by random primer R5 and oligo-dT anchored primer PT6. These two sequences had high similarities as the whole sequence of B20 was identical to the sequence of B15 with the exception of one base (T) at base position 61, which was replaced by C. Because of the similarity, the following homology analysis will use the sequence of B15 to represent both B15 and B20.

Fig. 4.3 cDNA sequences and similarities of B15 and B20, identical sequences are highlighted. B15R and B20 represent the reverse and forward sequences, respectively.

```
B15R    1    TGGATTGGTCGAAAAAGGATTCATCTCTGCCTATCTTTAC   40
B20     1    TGGATTGGTCGAAAAAGGATTCATCTCTGCCTATCTTTAC   40

B15R   41    AAACATAATGATGCATCATGTTCCTCGAGAGTCTCTAAAT   80
B20    41    AAACATAATGATGCATCATGCTCCTCGAGAGTCTCTAAAT   80

B15R   81    GAGCACAGGGTCCATGGTTAATTAACATGATCTACATCTT  120
B20    81    GAGCACAGGGTCCATGGTTAATTAACATGATCTACATCTT  120

B15R  121    CCTGTGCTCACCTGTAAAGTATTAAAACTCGGCAACATGT  160
B20   121    CCTGTGCTCACCTGTAAAGTATTAAAACTCGGCAACATGT  160

B15R  161    AAGTGGCAGTGAACTGCATCAGAGCATGTGTTCCGGTTTT  200
B20   161    AAGTGGCAGTGAACTGCATCAGAGCATGTGTTCCGGTTTT  200

B15R  201    GCTTCGGATGGAGAAGTGTATGTGAAGTTGTCATGTAACT  240
B20   201    GCTTCGGATGGAGAAGTGTATGTGAAGTTGTCTAAAAAAA  240

B15R  241    TGTGTTGATACTTTTAACACTTCTTCCTCA             270
B20   241    AAAAAA                                    246
```

B15 was significantly homologous to some barley cDNA clones from the dbEST of GenBank, the top three homologues are shown in Table 4.5. The identities of this transcript to the barley cDNA clones of AL509568, BF618484 and BF265514 were all 99 % over 226 bp of nucleotides, especially to AL509568 where just one different nucleotide within 259 bp was present. This result confirms that both B15 and B20 were expressed barley gene transcripts.

Table 4.5 The top three highly homologous EST sequences of B15.

GenBank Acc No	Characters	Identities
AL509568	*H. vulgare* Barke developing caryopsis	258/259 bp (99 %)
BF618484	*H. vulgare* seedling shoot cDNA clone	225/226 bp (99 %)
BF265514	*H. vulgare* seedling green leaf cDNA clone	224/226 bp (99 %)

Searching the nr nucleotide sequence database with the B15 sequence showed that it was homologous to *O. sativa* genomic DNA cds (AP00815), which was similar to the leucine zipper transcription factor *PNIL34* of *Ipomoea nil* (U37437). However, the identity was not satisfactory in that the identical region was only 22 bp and the E-value was ≥ 0.06, which is insufficient to be able to predict the function of B15 and B20. Searching the nr peptide sequence database with BLASTX with B15 found that this transcript was homologous to a human zinc finger protein (ZFD25) with low identity of 17/58 amino-acids (29 %). Again this identity is not high enough to indicate the function of B15 and B20 transcripts and the E-value was > 1. Because B15 showed 99 % homology to the barley ESTs AL509568 and BF265514 over 259 and 226 bp, respectively, this high identity suggests that they were probably the same transcript. Both AL509568 and BF265514 (659 bp and 651 bp, respectively) are substantially longer than B15 and B20. Using these longer EST sequences to search homologous sequences in GenBank could supply more information and suggest possible functions for both B15 and B20 transcripts. The search results with barley ESTs AL509568 and BF265514 from the nr nucleotide sequence database of GenBank

showed that they were highly homologous to *O. sativa* genomic DNA (AP00815) clones P0003H10 and P0025D05, which were similar to the leucine zipper transcription factor *PNIL34* of *I. nil* (U37437) and *A. thaliana* gene *At3g56940* encoding leucine zipper-containing protein AT103 (NP_191253, Table 4.6). BLASTX search results also showed that both AL509568 and BF265514 were homologous to leucine zipper-containing proteins of plants. BF265514 had higher identity than AL509568, therefore the homologues of the former are presented in Fig. 4.4. This EST transcript was highly homologous to the *Euphorbia esula* leucine zipper-containing protein AF417577 (the identity was 114/133 amino-acids, 85 %). It was also highly homologous to *I. nil* (U37437) and *A. thaliana* (T47754) leucine zipper-containing proteins. The identities were 109/133 (81 %) and 105/133 (78 %) amino-acids, respectively. These results are consistent in showing that either BF265514 or AL509568 is significantly homologous to leucine zipper transcription factors of different plant species, and suggested that the differential fragments B15 and B20 represented expressed barley leucine zipper transcripts.

Table 4.6 The top three genes homologous to AL509568 and BF265514 from the nr nucleotide sequence database of GenBank.

	AL509568	BF265514
1	AP000815 *O. sativa* genomic DNA, chromosome 1, clone:P0003H10, similar to *I. nil* leucine zipper transcription factor *PNIL34* (U37437), 229/267 bp, 85 %	AP000815 *O. sativa* genomic DNA, chromosome 1, clone:P0003H10, similar to *I. nil* leucine zipper transcription factor *PNIL34* (U37437), 230/266 bp, 86 %
2	AP001072 *O. sativa* genomic DNA, chromosome 1, PAC clone:P0025D05, similar to *I. nil* leucine zipper transcription factor *PNIL34* (U37437), 229/267 bp, 85 %	AP001072 *O. sativa* genomic DNA, chromosome 1, PAC clone:P0025D05, similar to *I. nil* leucine zipper transcription factor *PNIL34* (U37437), 230/266 bp, 86 %
3	NM_115553 *A. thaliana* gene *At3g56940* with product of leucine zipper-containing protein AT103 (NP_191253), 110/127 bp, 86 %	NM_115553 *A. thaliana* gene *At3g56940* encoding leucine zipper-containing protein AT103 (NP_191253), 165/200 bp, 82 %

Fig. 4.4 The homologous leucine zipper-containing proteins of *E. esula* (AF417577), *I. nil* (U37437) and *A. thaliana* (T47754) to barley EST BF265514; identical sequences are highlighted.

```
BF265514      3  WKAKLWSRFFCLSVYITMYLNDCQRSAFYEGIGLN  37
E.esula     273  WKAKLWARFFCLSVYVTMYLNDCQRTAFYEGIGLD 307
I.nil       238  WKAKLWARFFCLSVYVTMYLNDCQRTAFYEGIGLD 272
A.thaliana  277  WQAKLWSRFFCLSVYVTMYLNDCQRTNFYEGIGLN 311

BF265514     38  TKEFDMHVIYETNRTTARIFPAVPDVENPEFKRKL  72
E.esula     308  TKEFDMHVIIETNRTTARIFPAVLDVENPEFKRKL 342
I.nil       273  TKEFDMHVIIETNRTTARVIFPAVLDENPEFKRKL 307
A.thaliana  312  TKEFDMHVIIETNRTTARIFPAVLDVENPEFKRKL 346

BF265514     73  DRMVDINLKIISIGESNDLPLVKNLKRVPLIAQLV 107
E.esula     343  DRMVVINQKLQAVGETEDNSVVKNLKRVPLIAALV 377
I.nil       308  DRMVEINQKLLAVGETDDIPLVKNLKRIPLVAALV 342
A.thaliana  347  DRMVVSYEKLLAIGETDDASFIKTLKRIPLVTSLA 381

BF265514    108  SEIIAAYLMPPIESGSVDFAEFEPKLVY        135
E.esula     378  SEILAAYLMPPIESGSVDFAEFEPKLVY        405
I.nil       343  SEILAAYLMKPVESGSVDLAEFEPQLGY        370
A.thaliana  382  SEILAAYLMPPVESGSVDFAEFEPNLVY        409
```

4.3.3 Homology analysis of B22

The sequence of B22 (GenBank accession number: BF718809) was 216 bases long including a random primer R5 sequence at the 5' end and a PT6 antisense primer sequence at its 3' end (Fig. 4.5).

Fig. 4.5 cDNA sequence of B22.

R5 sense primer

```
1   TGGATTGGTCATAGCGTCTTTTGTAATATTGTCTCGTGTAATATC  45
46  ACCTCCTGTATTGTTGAACCAAAGCACTTGAGCTCTTTTTTGGTT  90
91  CCACTTCCCAGAACTGTTAGCAAGAGCATCCTAGGCCAAGATGCT 135
136 CGAACACGCTGGTTGTATCTACTTTTGTGTATGAATAAGGATGGG 180
181 GCAATAAACCGTGCTGGACTGCTAAAAAAAAAAAAAA         216
```

PT6 anti-sense primer

The search results from the dbEST of GenBank indicated that B22 was significantly homologous to barley ESTs (Table 4.7), *i.e.* 99 % similar to *H. vulgare* Barke root EST clones (AL502302 and AL501329) over 189 bases, and also 99 % homologous to another *H. vulgare* Barke root EST clone (AL502245). The cDNA sequence of this differential band was significantly homologous to vacuolar ATPase (V-ATPase) genes of several cereal plant species including barley (Table 4.7). The first homologue was the *H. vulgare* V-ATPase B subunit (L11862) with 98 % homology over 198 bases at the DNA level. B22 was aligned to the 3' untranslated region (3'UTR) of this V-ATPase B subunit gene. The other two homologues both represented the V-ATPase B subunit of *O. sativa* (AF375052 and AB055106), it was aligned to two separate sections with a 29 bp gap. The identities were 62/71 bp (87 %) and 41/46 bp (89 %). The rest of the matches were from human chromosome clones with very low homology. The E-value was > 0.013 and the identical region was just 23 bp or less. For example, the fourth best match was human chromosome 5 clone RP11-489L13 (AC022445) (23/23 bp, E-value = 0.013).

Table 4.7 The top three homologues for B22 from EST and DNA (nr) databases of GenBank. A 19 bp gap between the two identical regions in AF375052 and AB055106.

	GenBank Acc No	Characters	Identities
EST	AL502302	*H. vulgare* Barke roots cDNA clone	188/189 bp, 99 %
	AL501329	*H. vulgare* Barke roots cDNA clone	188/189 bp, 99 %
	AL502245	*H. vulgare* Barke roots cDNA clone	180/181 bp, 99 %
DNA	L11862	*H. vulgare* V-ATPase B subunit	196/198 bp, 98 %
	AF375052	*O. sativa* V-ATPase B subunit	62/71 bp, 87 % 41/46 bp, 89 %
	AB055106	*O. sativa* gene for V-ATPase B subunit	62/71 bp, 87 % 41/46 bp, 89 %

4.3.4 Homology analysis of B26

The sequence of B26 (GenBank accession number: BF718810) was 324 bases long including a PT4 oligo-dT primer at the 5' end and a PT4 antisense oligo-dT primer at the 3' end (Fig. 4.6).

Fig. 4.6 cDNA sequence of B26.

PT4 primer

```
1   TTTTTTTTTTTTTCGGATAAATAAGAAAAGGGTGCACGATCTCGA  45
46  CGAATTACTTCTGAATAAATTCAGAAATCATATGGAAGAACCATA  90
91  GCATTTCGCGACCTCATTGGTAAATCAATTTTGATTCTCTATAAA 135
136 CCAAGAATGTGAGACCATTAACACGGTTAAAGCTAAACTGCTTGA 180
181 AGTCCAGGCAAAAAGAGGTACTCTTTCTACAACTATATTAGTATT 225
226 AGTACCGAATTTAAACGGGAAATAGCTAATGTAAAATTTATCTGA 270
271 TATAGAACACTCATATCGATAAAATGGTTTGAACTATTTACGAAA 315
316 AAAAAAAAA  PT4 antisense primer                324
```

The BLASTN search results from the dbEST showed that the top three homologues of differential band B26 were all barley EST transcripts (Table 4.8). The first two transcripts BE438149 and BE437710 were leaf cDNA clones with a homology of 99 % within 298 bp. The top three homologues from the nr nucleotide sequence database showed that B26 was significantly homologous to a chloroplast gene (*atpF*) encoding ATP synthase (Table 4.8). The first homologue was the *H. vulgare* chloroplast gene *atpF* encoding ATP synthase CFO subunit I (AJ010573), the others were *T. aestivum* chloroplast gene *atpF* encoding ATP synthase CFO subunit I (NC_002762 and X02595). All of these showed the same significantly high identity of 99 % over 296/298 bp. B26 was aligned to the non-coding region of the gene *atpF*. In addition, B26 was also homologous to the chloroplast gene (*atpF*) of other plant species, *e.g. O. sativa* (X15901), *Zea mays* (X86563), *Nicotiana tabacum* (Z00044) and *Lotus japonicus* (AP002983).

Table 4.8 The top three homologous results from EST and DNA (nr) databases of GenBank with B26.

	GenBank Acc No	Characters	Identities
EST	BE438149	*H. vulgare* leaf epidermis cDNA clone	297/298 bp (99 %)
	BE437710	*H. vulgare* leaf epidermis cDNA clone	297/298 bp (99 %)
	BG309760	*H. vulgare* seedling shoot cDNA clone	239/240 bp (99 %)
DNA	AJ010573	*H. vulgare* chloroplast gene encoding ATP synthase CFO subunit I, exon 1 partial and exon 2	296/298 bp (99 %)
	NC_002762	*T. aestivum* chloroplast gene *atpF* encoding ATPase I subunit	296/298 bp (99 %)
	X02595	*T. aestivum* chloroplast gene encoding ATP synthase CFO subunit I	296/298 bp (99 %)

4.3.5 Homology analysis of differential band B12

The cDNA sequence of differential band B12 (GenBank accession number: BF718811) was 522 bases long (Fig. 4.7), this sequence has a R5 random primer and a R5 antisense primer at the 5' and 3' ends without a poly-A tail.

Fig. 4.7 cDNA sequence of B12.

R5 primer

```
1   TGGATTGGTCTGAGGAACGTCTTGAGATTCAGGCAATTGCGGATG  45
46  ATATAACTAGTAAATATGTTCCTCCCCATGTCAACATATTTTATT  90
91  GTTTAGGGGGAATTACACTTACTTGTTTTCTAGTACAAGTCGCTA 135
136 CCGGTTTTGCTATGACTTTTTACTACCGACCAACCGTTACAGAGG 180
181 CTTTTTCCTCGGTTCAATACATAATGACCGAGGCCAACTTTGGTT 225
226 GGTTAATCCGATCAGTTCATCGATGGCCAGCAAGTATGATGGTTT 270
271 TAATGATGATCCTGCATGTATTTCGTGTGTATCTCACAGGGGGAT 315
316 TTAAAAAACCCCGTGAATTAACTTGGGTGACAGGTGTGGTTTTGG 360
361 CTGTTTTGACTGCATCATTTGGTGTAACTGGTTATTCTTTGCCTT 405
406 GGGATCAAATTGGCTATTGGGCAGTCAAAATTGTGACAGGTGTAC 450
451 CTGACGCCATTCCGGTAATAGGATCGCCTTTAGTGGAGTTATTAC 495
496 GCGGAAGTGCTAGTGTGGACCAATCCA                    522
```

R5 antisense primer

The homologues of B12 obtained from the dbEST of GenBank showed that this differential band had 93 % identity (377/403 bp) with *Z. mays* leaf and shoot EST clone (BI396192), and also 88 % identity (441/499 bp) to two *Medicago truncatula* cDNA clones (BI312154 and BI264764) (Table 4.9). The three top homologues from the DNA (nr) database (Table 4.9) strongly suggested that the differential band B12 was a transcript of chloroplast gene *petB*. It showed 99 % homology (517/520 bp) to the *H. vulgare* chloroplast gene *petB* (X14107) and 98 % homology (514/520 bp) to the *T. aestivum* chloroplast gene *petB* (NC_002762 and X54751). It was further confirmed by matching the chloroplast gene *petB* of other plant species, *e.g. Z. mays* (S67283) (494/520 bp, 95 %), *O. sativa* (X15901) (494/520 bp, 95 %), *A. thaliana* (AP000423) (446/497 bp, 89 %), *Pinus thunbergii* (D17510) (443/496 bp, 89 %), *N. tabacum* (Z00044) (445/499 bp, 89 %), *Spinacia oleracea* (AJ400848) (442/499 bp, 88 %) and *M. truncatula* (441/499 bp 88 %). Search results in the nr peptide sequence database with BLASTX (Fig. 4.8) also showed that the putative protein sequence of B12 was highly homologous to barley, wheat, rice and maize plastoquinol-

plastocyanin reductase cytochrome b6, the similarity was 170 to 173 amino-acids (98 %). The rest matches were all cytochrome b6 from other plant species.

Table 4.9 The top three homologous results from dbEST and nr nucleotide databases of GenBank with B12.

	GenBank Acc No	Characters	Identities
EST	BI396192	*Z. mays* leaf and shoot cDNA	377/403 bp, 93 %
	BI312154	*M. truncatula* cDNA clone	441/499 bp, 88 %
	BI264764	Phosphate starved leaf *M. truncatula* cDNA clone	441/499 bp, 88 %
DNA	X14107	*H. vulgare* chloroplast gene *petB*	517/520 bp, 99 %
	NC_002762	*T. aestivum* chloroplast gene *petB*	514/520 bp, 98 %
	X54751	*T. aestivum* chloroplast gene *petB*	514/520 bp, 98 %

Fig. 4.8 The homology of the putative translated protein of B12 to plastoquinol-plastocyanin reductase cytochrome b6 of different cereal crops. BCyB6, RCyB6, WCyB6 and MCyB6 representing barley, rice, wheat and maize cytochrome b6, respectively.

```
B12        3 DWSEERLEIQAIADDITSKYVPPHVNIFYCLGGITLTCFL  42
BCyB6     23 DWFEERLEIQAIADDITSKYVPPHVNIFYCLGGITLTCFL  62
RCyB6     23 DWFEERLEIQAIADDITSKYVPPHVNIFYCLGGITLTCFL  62
WCyB6      6 DWFEERLEIQAIADDITSKYVPPHVNIFYCLGGITLTCFL  45
MCyB6     23 DWFEERLEIQAIADDITSKYVPPHVNIFYCLGGITLTCFL  62

B12       43 VQVATGFAMTFYYRPTVTEAFSSVQYIMTEANFGWLIRSV  82
BCyB6     63 VQVATGFAMTFYYRPTVTEAFSSVQYIMTEANFGWLIRSV 102
RCyB6     63 VQVATGFAMTFYYRPTVTEAFSSVQYIMTEANFGWLIRSV 102
WCyB6     46 VQVATGFAMTFYYRPTVTEAFSSVQYIMTEANFGWLIRSV  85
MCyB6     63 VQVATGFAMTFYYRPTVTEAFSSVQYIMTEANFGWLIRSV 102

B12       83 HRWPASMMVLMMILHVFRVYLTGGFKKPRELTWVTGVVLA 122
BCyB6    103 HRWSASMMVLMMILHVFRVYLTGGFKKPRELTWVTGVVLA 142
RCyB6    103 HRWSASMMVLMMILHVFRVYLTGGFKKPRELTWVTGVVLA 142
WCyB6     86 HRWSASMMVLMMILHVFRVYLTGGFKKPRELTWVTGVVLA 125
MCyB6    103 HRWSASMMVLMMILHVFRVYLTGGFKKPRELTWVTGVVLA 142

B12      123 VLTASFGVTGYSLPWDQIGYWAVKIVTGVPDAIPVIGSPL 162
BCyB6    143 VLTASFGVTGYSLPWDQIGYWAVKIVTGVPDAIPVIGSPL 182
RCyB6    143 VLTASFGVTGYSLPWDQIGYWAVKIVTGVPDAIPVIGSPL 182
WCyB6    126 VLTASFGVTGYSLPWDQIGYWAVKIVTGVPDAIPVIGSPL 165
MCyB6    143 VLTASFGVTGYSLPWDQIGYWAVKIVTGVPEAIPVIGSPL 182

B12      163 VELLRGSASVDQS                            175
BCyB6    183 VELLRGSASVGQS                            195
RCyB6    183 VELLRGSASVGQS                            195
WCyB6    166 VELLRGSASVGQS                            178
MCyB6    183 VELLRGSASVGQS                            195
```

4.3.6 Homology analysis of B28

The cDNA sequence of differential band B28 (GenBank accession number: BF718812) was 514 bases long including a R5 sense primer and a R5 antisense primer at the 5' and 3' ends (Fig. 4.9).

Fig. 4.9 cDNA sequence of B28.

R5 primer

```
1   TGGATTGGTCACAGGAAGCGAAAACTGCCCCGAGTATCTGGCCGA  45
46  TAAGAAGGCGGCCAATGCTAGATAAGGTATATTTGATATACATGT  90
91  TATTGATGTGTACCTCACCAGCTCTCGTAGTAGTGCTTCGGTATT 135
136 TGATACCGGTTCTATTGCTCACATTTGCAACTCGAAACAGGAACT 180
181 ATGGAATAAACAAAGGATGGCGAAGGATGAACTGACGATGCACGT 225
226 GGGAAATGGTTCCAAGGTTGATACGATCGCCGTCGGCACGATCTC 270
271 ACTTGAGTTACCATCAGGATTAGTGATGAACTTAAATAATTGCTA 315
316 TTTAGTGTGTGTGTTGAGCATGAACATTATATCTGGATCTTGTTT 360
361 ATTGCGAGACGATTACTCATTTAAATCAGAGAATAATTGTTGATC 405
406 TATTTATATGGGTAATATCTTTTATGGTCATGCACCCAATTTGAG 450
451 GGATTTGTTCATGTTGAATCTTGATAGTGATGAAACTCATATCCA 495
496 TAACATTGAGACCAATCCA                            514
```

R5 antisense primer

All top three homologues of B28 obtained from the dbEST of GenBank were from cereal plants (Table 4.10). The highest identical homologue was a *H. vulgare* EST (BG417806), which was 87 % homologous to B28 (386/442 bp). The other two homologies with BF264456 (barley) and BE425043 (wheat) were 85 % (196/228 bp) and 81 % (302/370 bp), respectively.

The top three homologues of B28 from the nr nucleotide database of GenBank showed that all of them were BARE-1 copia-like retroelements from *H. vulgare* (Table 4.10). The identity to the first homologue barley BARE-1 copia-like retroelement (Z17327) was 435/489 bp (88 %), to the second homologue AY013246, which is a part of *H.*

vulgare chromosome 5 BAC and for BARE-1 copia-like retroelement was 422/478 bp (88 %). The third was the *H. vulgare* retrotransposon BARE-1 for *gag*-protease fusion polyprotein precursor (AJ295226), the identity was 261/297 bp (88 %). These results suggest that the differential band B28 was a transcript of the BARE-1 copia-like retroelement. Further search results from the nr peptide sequence database of GenBank also produced similar results (Table 4.10). The top three homologous protein matches of the deduced peptide sequence of B28 were *Oryza australiensis* polyprotein (BAA22288) with an identity of 74/164 amino-acids (45 %), *A. thaliana* putative transposon protein (CAB77943) with an identity of 56/142 amino-acids (39 %), and an *A. thaliana* protein showing similarity to retrovirus-related polyproteins (AAD17359) with an identity of 43/129 amino-acids (33 %), respectively. Beside the homology to retroelement polyprotein, there were two other homologues, one was *A. thaliana* lectin receptor kinase (CAA29271) (28/117 amino-acid, 23 %, E-value = 0.067), the other was *Caenorhabditis elegans* probable G protein-coupled receptor B0563.6 (Q11082) (17/58 amino-acid, 29 %, E-value = 2.8), but the identities to these two homologues were very low.

Table 4.10 The homologues of B28 from the dbEST, nr nucleotide and peptide sequence databases of GenBank.

	GenBank Acc No	Characters	Identities
EST	BG417806	*H. vulgare* cDNA clone	386/442 bp, 87 %
	BF264456	*H. vulgare* cDNA (*Blumeria* challenged)	196/228 bp, 85 %
	BE425043	Wheat endosperm cDNA library *T. aestivum*	302/370 bp, 81 %
DNA	Z17327	*H. vulgare* DNA for BARE-1 copia-like retroelement	435/489 bp, 88 %
	AY013246	*H. vulgare* chromosome 5 BAC, BARE-1 copia-like retroelement	422/478 bp, 88 %
	AJ295226	*H. vulgare* retrotransposon BARE-1 partial pseudogene for *gag*-protease fusion polyprotein precursor	261/297 bp, 87 %
Protein	BAA22288	polyprotein [*Oryza australiensis*]	74/164 amino-acids, 45 %
	CAB77943	putative transposon protein [*A. thaliana*]	56/142 amino-acids, 39 %
	AAD17359	similarity to retrovirus-related polyproteins [*A. thaliana*]	43/129 amino-acids, 33 %

4.3.7 Homology analysis of B14, B8/B13/ B16, B9 and B27

The differential bands B14, B8/B13/B16, B9 and B27 had similar sequences while B8, B13 and B16 had the same sequence, which was presented as B8/B13/B16 (Fig. 4.10). The sequence of B8/B13/B16 (GenBank accession number: BF718814) was 307 bases long. The cDNA sequence of B9 (GenBank accession number: BF718815) was 309 bases long. The sequences of B8/B13/B16 and B9 had a R5 random primer at the 5' end and a PT6 anti-sense oligo-dT primer at the 3' end, both sequences possess another repeated R5 sequence, which was located 16-18 bases away from the first random primer sequence (R5). Most of the B9 sequence was identical to B8/B13/B16 except for 18 bases between the two R5 primers. The differential band B14 (GenBank accession number: BF718813) was 252 bases long, there was no primer sequence at its 3' end. It was observed that there were three repeated R5 random primer sequences at the 5' end and between them was a short intervening sequence. The sequence of B14 after the second R5 site was identical to B8/B13/B16 except for being 81 bases short at the 3' end. Another feature of this sequence is that the first R5 sequence and the following 16 bases were identical to the second R5 site and the following 16 bases except for one base difference. The cDNA sequence of B27 (GenBank accession number: BF718821) was 305 bases long including both R5 and PT6 primers at the 5' and 3' ends, respectively even though the poly-A tail was very short with just two adenine bases. The sequence of B27 was identical with B8/B13/B16 with the exception of part of the sequence of the first R5 and the following 24 bases. It is clear that the differences among these four differential transcripts are all from the 5' end of the transcript before the last R5 sequence.

Fig. 4.10 cDNA sequences of B9, B8/B13/B16, B14 and B27 (sequence similarities are highlighted and primer sequences are underlined).

```
B14              1                                          TGGATTGGTCGTAGATGT  18

B9               1          TGGATTGGTCGTAGGGTGTCGGCGGTAATGGATTGGTCGTG  41
B8/B13/B16       1            TGGATTGGTCGTAGATGTGTTCGCTCTGGATTGGTCGTG  39
B27              1     TGGATTGGTCTGTACATTGTCATCTCGTGTTGTATGGATTGGTCGTG  47
B14             19     GTTCGCTGTGGATTGGTCGTAGATGTGTTCGCTCTGGATTGGTCGTG  65

B9              42     GGATCTACAACATGTCTAACGGGATCGGGAAAAAGGAGTGAATTTCT  88
B8/B13/B16      40     GGATCTACAACATGTCTAACGGGATCGGGAAAAAGGAGTGAATTTCT  86
B27             48     GGATCTACAACATGTCTAACGGGATCGGGAAAAAGGAGTGAATTTCT  94
B14             66     GGATCTACAACATGTCTAACGGGATCGGGAAAAAGGAGTGAATTTCT 112

B9              89     GTCGGTTTTGCTAGTATCTCAGGAAGCGGCATGGAAGCGATACTGCC 135
B8/B13/B16      87     GTCGGTTTTGCTAGTATCTCAGGAAGCGGCATGGAAGCGATACTGCC 133
B27             95     GTCGGTTTTGCTAGTATCTCAGGAAGCGGCATGGAAGCGATACTGCC 141
B14            113     GTCGGTTTTGCTAGTATCTCAGGAAGCGGCATGGAAGCGATACTGCC 159

B9             136     CAAGCTATGATGCCATCTTCGTTTGCAAATAATATTGTCAGAGAAAG 182
B8/B13/B16     134     CAAGCTATGATGCCATCTTCGTTTGCAAATAATATTGTCAGAGAAAG 180
B27            142     CAAGCTATGATGCCATCTTCGTTTGCAAATAATATTGTCAGAGAAAG 188
B14            160     CAAGCTATGATGCCATCTTCGTTTGCAAATAATATTGTCAGAGAAAG 206

B9             183     ACTGAGTTCATTTCCAGTTCTCTTTTGTTGGTATTTGTGGATATTTG 229
B8/B13/B16     181     ACTGAGTTCATTTCCAGTTCTCTTTTGTTGGTATTTGTGGATATTTG 227
B27            189     ACTGAGTTCATTTCCAGTTCTCTTTTGTTGGTATTTGTGGATATTTG 235
B14            207     ACTGAGTTCATTTCCAGTTCTCTTTTGTTGGTATTTGTGGATATTT  252

B9             230     ATGTCAGCAAATTGATGCTAAACTGGCAATGATGATTCTATAACATT 276
B8/B13/B16     228     ATGTCAGCAAATTGATGCTAAACTGGCAATGATGATTCTATAACATT 274
B27            236     ATGTCAGCAAATTGATGCTAAACTGGCAATGATGATTCTATAACATT 282

B9             277     GGCATCATTATCCTTTCTGCTAAAAAAAAAAAA               309
B8/B13/B16     275     GGCATCATTATCCTTTCTGCTAAAAAAAAAAAA               307
B27            283     GGCATCATTATCCTTTCTGCTAA                         305
```

The top three EST homologues for each search are listed in Table 4.11. These four differential sequences showed high homology to *H. vulgare* EST clones. For example, B8/B13/B16, B9 and B27 were 100 % (265/265) identical to *H. vulgare* testa/pericarp EST BG414877, and B14 was also 100 % (198/198) identical to barley EST clones (BG416347, BG414877 and AL450730). The results indicated that these isolated differential fragments are expressed barley gene transcripts. Search results from DNA (nr) and protein databases did not supply satisfactory homologues, the best match was to *Mus musculus* plasmid rescue fragment AF269239 (21/21 bp, E = 0.003) at DNA level and *A. thaliana* unknown protein AF424552 (15/16 amino-acids, E = 0.19). So the functions of differential bands B9, B8/B13/B16, B14 and B27 are unclear.

Table 4. 11 EST homologues of B8/B13/B16, B9, B14 and B27.

	B8/B13/B16	B9	B14	B27
1	BG414877 *H. vulgare* testa/pericarp cDNA clone 265/265 (100 %)	BG414877 *H. vulgare* testa/pericarp cDNA clone 265/265 (100 %)	BG416347 *H. vulgare* testa/pericarp cDNA clone 198/198 (100 %)	BG414877 *H. vulgare* testa/pericarp cDNA clone 265/265 (100 %)
2	AL450730 *H. vulgare* Barke etiolated leaves cDNA clone 265/265 (100 %)	AL450730 *H. vulgare* Barke etiolated leaves cDNA clone 265/265 (100 %)	BG414877 *H. vulgare* testa/pericarp cDNA clone 198/198 (100 %)	AL450730 *H. vulgare* Barke etiolated leaves cDNA clone 265/265 (100 %)
3	BF625249 *H. vulgare* seedling shoot cDNA clone 264/265 (99 %)	BF625249 *H. vulgare* seedling shoot cDNA clone 264/265 (99 %)	AL450730 *H. vulgare* Barke etiolated leaves cDNA clone 198/198 (100 %)	BF625249 *H. vulgare* seedling shoot cDNA clone 264/265 (99 %)

4.3.8 Homology analysis of B17, B19, B24 and B25

The cDNA sequence of B17 (GenBank accession number: BF718819) was 329 bases long with a R5 random primer at the 5' end and an R5 antisense primer at its 3'end (Fig. 4.11a). B17 was homologous to a *Fusarium* infected barley (*H. vulgare*) EST clone (BI950088) with 55/55 bp (100 %) identity. Band B19 (GenBank accession number: BF718818) had 338 bases with an R5 primer and a PT6 antisense primer sequence at the 5' and 3' ends, respectively (Fig. 4.11b). This transcript was significantly homologous to a dehydration stressed *H. vulgare* EST clone (BG300228) with an identity of 271/272 (99 %). Bands B24 and B25 (GenBank accession numbers: BF718817 and BF718816) were 347 and 363 bases long, respectively (Fig. 4.11c,d),

both sequences were characterised by a PT4 sense primer and a PT4 antisense primer at the 5' and 3' ends, respectively. B24 was homologous to a *H. vulgare* EST (AW982886) with an identity of 113/119 bp (94 %), B25 was homologous to a *T. aestivum* EST clone (BE430176) with an identity of 185/204 bp (90 %). The above results indicate that all these four differential bands were expressed gene transcripts, which were either homologous to *H. vulgare* ESTs or *T. aestivum* ESTs from the dbEST of GenBank. However, no significant homologous matches were obtained for these transcripts from the present nr nucleotide and peptide sequence databases of GenBank and other databases. The best homology to B17 at the DNA level was the human clone RP11-113H14 of chromosome 8 (AC021236) (21/21 bp) and there were three matches at the protein level, which were *A. thaliana* xylulose kinase (BAA97229) (16/28 amino-acids, 57 %), *C. elegans* protein similar to vacuolar membrane protein PEP3 (AAC46819) (12/38 amino-acids, 31 %) and *Rickettsia conorii* DNA-binding protein HU (NP_359849) (22/77 amino-acids, 28 %). The search results of B19 showed that the best match at DNA level was *Sulfolobus tokodaii* gene *ST2235* (AP000989) encoding 285 amino-acids long hypothetical dihydrodipicolinate. synthase (20/20 bp). At the protein level, B19 matched four homologues, three were *Plasmodium falciparum* calcium-dependent protein kinase (AF106064, CAB11118, NP_473217) (14/38 amino-acids, 36 %) and one was *S. tokodaii* 248 amino-acids long hypothetical protein (BP_378473) (16/53 amino-acids, 30 %). It is possible that B19 is related to calcium-dependent protein kinase. From the nr nucleotide database the best match of B24 was *P. falciparum* clone HB3 falcipain 2 gene (AF282979) (23/24 bp, 95 %), and from nr peptide database it aligned to *Neurospora crassa* hypothetical protein 15E6.200 (T48812) (19/58 amino-acid, 32 %) and *A. thaliana* putative protein kinase (NP_178651) (16/50 amino-acids, 32 %). B25

also showed poor homology both at DNA and protein levels, its best homologues from nr nucleotide and peptide databases were human DNA clone RP1-249I4 (AL512303) (21/21 bp) and *O. sativa* putative Rad51C protein (BAB32931) (11/34 amino-acids, 32 %). So the above identities could not predict functions of the differential bands B17, B19, B24 and B25.

Fig. 4.11 cDNA sequences of B17, B19, B24 and B25.

R5 primer

a B17

```
   1 TGGATTGGTCTTTCCCGTTGCAGTATGTTATCACATTTGC  40
  41 TTTCAGCACATATGAATTAGTAACAATTTGAATGGATTAC  80
  81 TACTGTCTCAACTATAAATTTTCCAGGCCTGCTAACCTTT 120
 121 CTGTAGTTTTGTCGTTGGTTAAAAACTTGCTCATGCTGAT 160
 161 TTTCACCATGACAGTGAATGAAGTGTGACTCTTAAGTTTT 200
 201 TGACATAATGAAATTCATCTTTTTTTTCATATAGGTGGTA 240
 241 GGTTAGGATTCTACTACAAAGACCATGAAATCCTGCCTCC 280
 281 TCTTCCAGGTAATCATGTAATAGTGTTTTTAAGTTTTTTG 320
 321 ACCAATCCA                                 329
```

R5 antisense primer

R5 primer

b B19

```
   1 TGGATTGGTCGTTGTGATTGAATGGCCGCTTGTGGAATAA  40
  41 TACAAGGGATGAATGAACTTGATAGTTCGTAGGTCGTTAA  80
  81 ATTTTAGTGTTGCGCCAGCTATGATCTGCATCCGTGGATG 120
 121 CTCTAGTTGCCTGCTAGAGTACCAGGGTTAGTTTACCTTT 160
 161 ATATCCTGGCTCCGCTGGTCTACGTTAGCATTTGGCTATA 200
 201 TACCCCTGCCCAGTAATGGTCATGATCAAGTATGCTATTT 240
 241 TGGACTGTTCTAAATCGTAAGAATTCAAGTTACTGGTGAT 280
 281 TCTTACCGAGTTCAACCTAAAAAAACAACCAAGCTTAAGT 320
 321 GTGCTAAAAAAAAAAAAA                        338
```

PT6 antisense primer

PT4 primer

c B24

```
   1 TTTTTTTTTTTTCGGGGGGGGGGGGGGGGGGGGTTACTTT  40
  41 TTCTGGTAGCTTCGGTGTTTAGTCCACATTTGAACTAAAT  80
  81 ACCAAAACTCTCCAGAGGAATACTGCCCCATATCTGAACA 120
 121 GGCTCTTGGCATGTATGTACATTATTTGGTGTCGGTGGTG 160
 161 CGAGGTTTTGTTAGCGTGCAAAATATGGCTATTCGTTTAT 200
 201 GCCTTTGTTGTATTGGATGGTGTGTTTTCTTTAATTTATA 240
 241 CCCATGTTCGCTTCTTGAAAGAATGATGGGAAGATTTATT 280
 281 TGGTCGTGTCCCACCTGTTAACGCCAATGAAGTTGAGTTG 320
 321 ATCTCCCATTTGGCGAAAAAAAAAAAA               347
```

PT4 antisense primer

PT4 primer

d B25

```
   1 TTTTTTTTTTTTCGCCTAGAGCCTATTTATACAGCCTCAG  40
  41 TGACCCTGTACAAATAGCAAAGTATCAAGTCAAACGACAA  80
  81 AGTTCCAAGTTTTGCCAGTAGCTGGTATTACCGGCGATTC 120
 121 CGAAAATTTCCGACAATGTAATGCTGAGCTACAAATTTGC 160
 161 TCCAGCTCAGCATGAGCCTGATCCAGGTGTAAGTAGCACC 200
 201 AGAAAGTTTCAGTTGTTGTAATGGCACTAATTCCTAGGAC 240
 241 TTGAACTCAATGAAGCAATTTTTTCATTACAGTCCTCAAG 280
 281 TGTTCCAGATCACAACGATTCCAGAGTATTACCTGTGTCG 320
 321 GTGTGTAATAAAGATGACATTTTGAGTCTCGAAAAAAAAA 360
 361 AAA                                      363
```

PT4 antisense primer

4.3.9 Homology analysis of B11, B18 and B21

The cDNA sequences of B11, B18 and B21 (GenBank accession numbers: BF718822, BF718820 and BF718823) were 432, 350 and 268 bases long, respectively (Fig. 4.12a, b, c). The main characteristics of these sequences were that: B11 had a R5 primer and a PT6 antisense primer at the 5' and 3'ends, respectively; B18 had no poly-A tail and this sequence was amplified by a R5 random primer and its antisense primer while B21 had a PT6 sense primer at the 5' end and a R5 antisense primer at the 3' end. No significant homologues were matched to these transcripts from the EST, DNA (nr) and protein databases of GenBank. The best matches to B11 were human BAC clone RP11-343N14 (AC006461) (24/25 bp, E-value = 0.43) and the human profilaggrin (A35938) (20/53 amino-acids, E-value = 0.85); to B18 were *Drosophila melanogaster* 3L BAC clone RP98-17D14 (AC010011) (23/23 bp, E-value = 0.022) and *Pyrococcus furiosus* hypothetical protein (NP_577759) 23/75 amino-acids, E-value = 1.7); to B21 was the human BAC clone RP11-26B22 (AC009299) (21/21 bp, E-value = 0.26), no match was found at the protein level. The above identities were not high enough to predict the functions of B11, B18 and B21.

Fig. 4.12 cDNA sequences of B11, B18 and B21.

R5 primer

```
a B11      1 TGGATTGGTCTGGTTTGAAAATCGTTGCTAGAGGAAGTAT  40
          41 GCATCACCTATTTGGTAGATGATGCATGGTAGTGCTATCT  80
          81 CTGAGCAATGGAGAAATAAAAAAGAGAGGGCCTTCCGCCT 120
         121 GTGTTGCATGAGCGACCTGCCTGCTCATGCTGCGGATTTG 160
         161 CCCTTGGTCTTCATTTGTGTGTTGTGGTGTGTGATCTGTC 200
         201 AGCTACAGACTGATTATTGTAATGCTAAGAAATTCCTTCG 240
         241 ATTTGCATGAGCGACCTGCCTGCTCATGCTGCGGATTTGC 280
         281 CCTTGGTCTTCATTTGTGTGTTGTGGTGTGTGATCTGTCA 320
         321 GCTACAGACTGATTATTGTAATGCTAAGAAATTCCTTCGA 360
         361 TTGTTGTACAAATGGTGATTTGTGCATGTCATCATATATA 400
         401 AGCTGCAGCTTTTTACGTCTAAAAAAAAAAAAA        432
```

PT6 antisense primer

R5 primer

```
b B18      1 TGGATTGGTCTATCGTTCCGCGGTAGATCTCTTGGAGATG  40
          41 GATGTCTATGGTCCCGAATATCTCATCACGAGAAGTGCCA  80
          81 TCGCGCCTCGAAATGCAAAATAAATCAACGCACCAAGGAT 120
         121 GCTTGTTCTGGCGAGTCGCATCCAATCAGAACGATTCTGT 160
         161 AGGAACCAGAATATATCGCTCGATTGGATATTGCCTTCAG 200
         201 AATTGATATTTCTATTTTTCCTGATGAATTCAAGATAGAT 240
         241 AGGAGGAAGGTATTTCGCCAACGGAGCGATAGGTTGATGA 280
         281 AAGATATCAATCGGCAGATGCTTTAAGAGTGGGTGCTCAT 320
         321 GACGAATTTTCTCAGCATCAGACCAATCCA          350
```

R5 antisense primer

PT6 primer

```
c B21      1 TTTTTTTTTTTTTAGTAGATTAGTAGTTCACTAAAGGGAAC  40
          41 AAACAAAACATGTCTTGTTCTCGCTGATAATAAGATATTT  80
          81 GCAGAGAACAAGAATCAATCAAAACCACACTCCATGAGGC 120
         121 AGGATAGATTAGCTAAAAAATTTAGCAACAAATGTGTACA 160
         161 CCAAAGCAACCAAATGAGGAACGCTGTTTTTAGAACATGA 200
         201 AGTCTGATGCCGAATCAATTTAAGGAAGCTCTCTCATTAG 240
         241 ACCTCTTGAACTCTTATGGACCAATCCA              268
```

R5 antisense primer

4.4 Discussion

This discussion will focus on short-term and long-term salt stress treatments with the control(s) and the expression of gene transcripts in relation to Northern blot analysis.

Using the mRNA differential display technique, 21 cDNA fragments between Golden Promise and Maythorpe in relation to salt stress were cloned. To assign possible functions to them, searching homologues from GenBank using computer program BLAST 2.0 (Altschul *et al.*, 1997) was useful and efficient. The GenBank sequence database incorporates publicly available DNA sequences of more than 105000 different organisms (Benson *et al.*, 2002). BLAST is used to identify similarities of a query sequence among sequences within a database. Claverie (1997) demonstrated that this computational method currently locates more than 80% of the internal coding exons. BLASTN queries a nucleotide sequence against a nucleotide database and BLASTX translates a nucleotide query into all six frames, three frames from each DNA strand, and searches a protein database for coding exons. The most abundant and easily obtainable gene prediction data are the ESTs found in dbEST database (Fortna and Gardiner, 2001). EST databases constitute an effective general-purpose probe for gene detection in genomic sequences by use of straightforward similarity search techniques. It is possible to detect more than 80 % of all known genes in genomic sequences (Bailey *et al.*, 1998). EST sequence is a decent indicator in the prediction of a gene (Fortna and Gardiner, 2001). According to Peri *et al.* (2001), a simple method of determining tissue specificity and approximate levels of expression of a given gene is to merely survey the dbEST for the presence of transcripts - a so-called 'electronic northern'. Using BLASTN searching facility, 18 out of 21 differential fragments showed high homology to barley (17) and maize (1) cDNA clones. Their identities

were more than 95 %, which indicated that these fragments were expressed gene transcripts. Ten differential bands were highly homologous to genes with known functions and the remaining eleven bands did not produce significant matches to any known genes by BLASTN and BLASTX searches. The cDNAs isolated by RT-DD were short (most of them were less than 500 bp), and in theory, these sequences derive from the 3' ends of poly-A tail and contained untranslated sequences (3'UTR). Some sequences extended far enough to reach and overlap with exons, most likely these sequences can match genes with identified functions, *e.g.* B1. Some of the clones might not be long enough to reach coding regions or conserved regions of a gene of sufficient length, or their corresponding genes have not been identified. Therefore no satisfactory homologues could be matched to predict functions of these transcripts, *e.g.* B14 and B27. Asamizu *et al.* (2000) also reported that from 12028 nr groups of the 3' end ESTs of *A. thaliana,* 4816 groups showed similarity to genes of known function, 1864 to hypothetical genes, and the remaining 5348 were novel sequences (the percentage of novel sequences was 44.5 %). In the present study, the percentage of novel sequences was 52.4 %, which was quite similar to the above result.

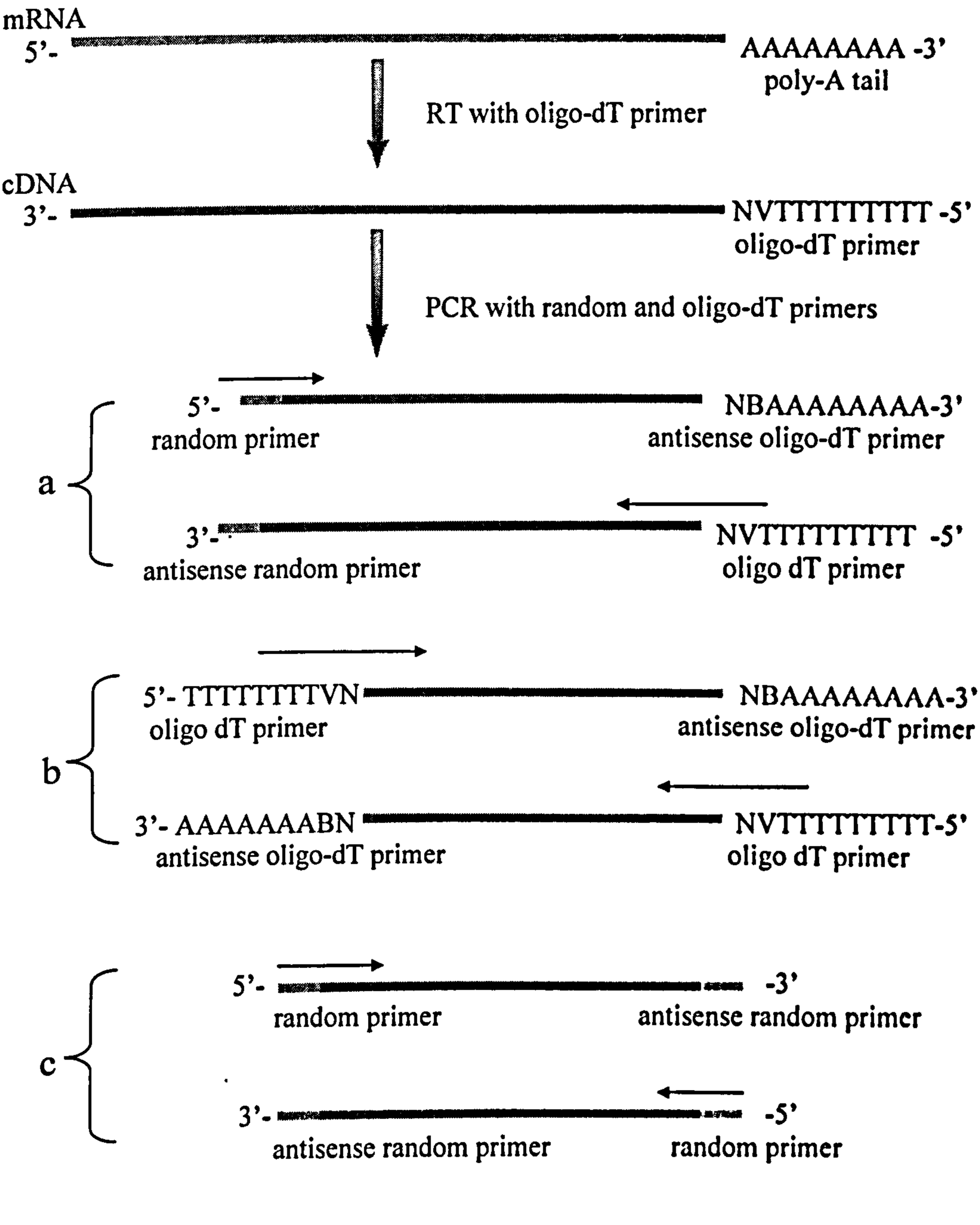
Some transcripts showed high similarities to others but were complementary sequences, such as B15 to B20, B14 and B27 to B9 and B8/B13/B16. These sequences could be produced by PCR amplification. It is also possible that some of them might be antisense transcripts, but further experiments are required to confirm that. A few cases of antisense transcripts have been reported in plants. For example, both sense and antisense transcripts for the *Bz2* gene in maize have been reported (Schmitz and Theres, 1992). The presence of antisense transcripts of the *MuDR* element in maize has been reported and confirmed by *in situ* hybridisation (Hershberger *et al.*, 1995;

Joanin *et al.*, 1997). Antisense RNA can regulate the expression of sense transcripts either as an enhancer or silencer (Terryn and Rouzé, 2000).

The cDNA sequences isolated by RT-DD in this study showed different structures related to the alignment of random and oligo-dT anchored primers to the 5' and 3' end. Some sequences have a random primer at the 5' end and an antisense oligo-dT primer at the 3' end while some have reverse sequences with an oligo-dT primer at the 5' end and an antisense random primer at the 3' end. In addition to this, some sequences have a random primer at the 5' end and an antisense random primer at the 3' end, whereas some sequences have an oligo-dT primer at the 5' end and an antisense oligo-dT primer at the 3' end. These differences can be explained by Fig. 4.13. Initially mRNA was reverse transcribed to the first strand of cDNA that contains an oligo-dT primer sequence at the 5' end. This cDNA was then used as a template to carry out PCR with a decamer random primer and an oligo-dT anchored primer. Double stranded DNA was thus created. There are three possible sequences that can be cloned from the consequent PCR products. One could be amplified by the above two primers, so that the cloned sequences could be a random primer at the 5' end and an antisense oligo-dT primer at the 3' end such as B3, or an antisense oligo-dT primer at the 5' end and an antisense random primer at the 3' end such as B21. Another type of sequence could only be amplified by the oligo-dT anchored primer, so the 5' and 3' ends of the sequence would be a sense oligo-dT primer and an antisense oligo-dT primer, respectively. The third option in terms of type of sequence represents a random primer annealed to the sequence and an antisense random primer annealed at the 3' end before the poly-A tail resulting in PCR amplified sequences with a sense random primer at the 5' end and an antisense random primer at the 3' end. This last kind of sequence

could represent both forward and reverse fragments. All these types of sequence could be the products of differential display.

Fig. 4.13 Possibilities of sequence structure of differential fragments following PCR. N = G, C, T, A; B = G, T, C; V = G, A, C.



Some differential fragments were exactly the same sequence, such as B1/B4, B5/B23 and B8/B13/B16 although they were identified from different positions on a polyacrylamide gel. On the polyacrylamide gel the size of these fragments might be different, but during PCR amplification after the bands were excised from the gel, random primers could re-align to the same position among the fragments resulting in the same sequences.

There were three consensus sequence groups, which are group one including B1/B4, B2, B3, B5/B23, group two including B15 and B20 and group three including B9, B8/B13/B16, B14 and B27. These bands shared their sequence similarities within each group. From the first two groups, the cDNAs shared most of the sequence within each cluster, major differences occurring from the 3' end where the poly-A signal was located. The results showed different splicing patterns and alternative splicing may play an important role in the diversity of mRNA (Mironov *et al.*, 1999; Croft *et al.*, 2000; Coward *et al.*, 2002). Environmental stress could cause alternative splicing. Cold stress induced exon skipping in a potato invertase gene transcript (Bournay *et al.*, 1996). Nissim-Rafinia and Kerem (2002) suggested that changes in the level of normal transcripts or in the relative pattern of different mRNA isoforms affect disease expression. So alternative splicing of pre-mRNA resulted in more differential mRNA species, which could be responses to salinity. In comparison with group three sequences, the differences among them were from the 5' end, where repeated the R5 random primer aligned to the repeated sections of the sequences.

Three photosynthesis related genes were differentially expressed between Golden Promise and Maythorpe. One gene encoded the small subunit of Rubisco (ribulose-

1,5-bisphosphate carboxylase/oxygenase) (*rbcS*) and related to differential fragments B1/B4, B2, B3 and B5/B23. Rubisco is an important component in plant photosynthesis, as the enzyme catalyses the CO_2 fixation reaction. It consists of two sets of subunits: the large subunits, which are encoded in the chloroplast genome and the small subunits, which are encoded in the nucleus as precursors and are then imported into the chloroplast and processed (Hwang and Tabita, 1989). The small subunit composition of Rubisco may differ in response to the environment (Goldschmidt-Clermont and Rahire, 1986). Northern blot analysis showed that B1 (homologous to Rubisco small subunit) was over-expressed in Maythorpe compared to Golden Promise under control conditions (Chapter 3). This may also be related to differences of growth between the two cultivars. Maythorpe is a comparatively tall plant and normally it grows faster than Golden Promise, hence it requires more energy, which is mainly produced from photosynthesis, for its development. Over-expression of the Rubisco small subunit could be one of the consequences. Expression of B1 was down-regulated by high salinity in Maythorpe, especially under short-term stress. Therefore, salt stress caused a serious reduction of the mRNA transcripts for the Rubisco small subunit in Maythorpe. As a result, photosynthesis could be reduced and in turn plant growth and development would be affected (Forster *et al.*, 1994). However, in Golden Promise this gene was induced and over-expressed under short-term salt stress but not in response to long-term salt stress. Short-term salt stress would result in a loss of turgor of cells and organelles including chloroplasts. As a response, the synthesis of osmotic solutes like glycine betaine, which are important to mechanisms of salt tolerance in barley, would be required (Grumet and Hanson, 1986). Salt tolerance was improved by the accumulation of glycine betaine in transgenic tobacco and proline in *Arabidopsis* (Liu and Zhu, 1997; Holmström *et al.*,

2000). Increased expression of the Rubisco small subunit in Golden Promise under salt stress would have an important role in the contribution of precursors for the synthesis of compatible solutes, which could be important in reducing the effect of salt shock compared to Maythorpe. Under long-term stress, the adaptation of expression of B1 to high salinity was different between the two cultivars. Golden Promise returned to normal with very low expression whilst Maythorpe maintained lower expression than in the control. This could imply that the photosynthesis of Maythorpe could be more markedly affected by salt than Golden Promise under long-term salt stress based on the down-regulation of the Rubisco small subunit. Maythorpe was not able to regulate Na^+ in shoot (Chapter 2), especially in young leaves, which could induce a reduction of enzyme activity including Rubisco.

The other two genes related to photosynthesis cloned in this study were a barley chloroplast gene (*petB*) for cytochrome B6 (corresponding band: B12) and a barley chloroplast gene (*atpF*) encoding ATP synthase CFO subunit I (corresponding band: B26), both of which are related to energy production in the chloroplast. Cytochrome B6 is located between photosystem I (PS I) and photosystem II (PS II) in the electron transport chain of the chloroplast. The major function of cytochrome B6 is to transport the electrons produced in the light reaction centre of PS II to the Fe-S protein in PS I to produce NADPH. The major function of ATP synthase is to produce ATP. Both NADPH and ATP are essential for CO_2 fixation (Salisbury and Ross, 1992). From Northern blot results, the over-expression of cytochrome B6 and ATP synthase in Maythorpe compared to Golden Promise under control conditions is consistent with that of the Rubisco small subunit. Both short-term and long-term salt stress caused down-regulation of the expression of cytochrome B6 in Maythorpe. In contrast, this

gene was induced and over-expressed under both salt treatments in Golden Promise. These results could suggest that when both cultivars were exposed to high salinity, Golden Promise increased the production of both NADPH and ATP whereas Maythorpe reduced these products, thereby having different effects on metabolism in the two cultivars.

Six different sized mRNA transcripts were hybridised to B26. The expression of each of them was low and not obviously affected by salt in Golden Promise compared to Maythorpe except for the biggest transcript (3.5 kb), which was slightly increased by both short-term and long-term salt stresses compared to its control. The expression of all the six transcripts in Maythorpe was down-regulated and significantly reduced by salt stresses compared to the control. Therefore, the gene encoding ATP synthase CFO subunit I was not sensitive to salinity in Golden Promise, but it was very sensitive to salt in Maythorpe. The obvious reduction of the expression of ATP synthase CFO subunit I in Maythorpe could result in a reduction in ATP production and hence growth.

A gene encoding V-ATPase B subunit was differentially expressed between the two cultivars in relation to salt stress, the corresponding fragment was B22. V-ATPase plays an important role in generating the electrochemical proton gradient across the tonoplast membrane, which can provide the driving force for the secondary transport of other ions and metabolites (Barkla *et al.*, 1999). This enzyme has three major subunits A, B, and c. Subunit B contains a nucleotide-binding site (Manolson *et al.*, 1985; DuPont *et al.*, 1990) and is considered to have a regulatory function. Therefore, altered expression of the subunit B gene could affect the expression of subunits A and

c. V-ATPase A and c subunits were reported to be induced by salinity in sugar beet and *Tortula ruralis* (Lehr *et al.*, 1999; Chen *et al.*, 2001). From Northern blot analysis, two RNA transcripts of V-ATPase subunit B (about 1.9 and 1.4 kb, respectively) were hybridised by B22. Both transcripts were over-expressed in Maythorpe compared to Golden Promise under control conditions, however the mechanism remains unknown. A possible reason could be that Maythorpe is a tall plant with a large leaf area, which undergoes rapid cell expansion requiring a large amount of water and ions to partition into the vacuole. Hence high expression of V-ATPase genes may be essential to maintain the electrochemical gradient across the tonoplast membrane in Maythorpe. Under short-term salt stress the expression of V-ATPase B was different between Golden Promise and Maythorpe compared to their controls. The expression in Maythorpe was reduced to a very low level, especially for the 1.4 kb transcript. This may be caused by Na^+ ions flowing into the vacuole in a short period resulting in disturbance of the electrochemical gradient across the tonoplast membrane and causing a reduction in expression of V-ATPase subunit B. In contrast, Golden Promise expressed more V-ATPase B transcripts than Maythorpe under short-term salt stress and it was up-regulated compared to the control treatment. This increased expression of V-ATPase B may be important for Golden Promise to maintain the electrochemical gradient across the tonoplast and reduce the effects of salt shock. Under long-term salt stress, the expression of V-ATPase B in Maythorpe recovered to some extent, especially the 1.4 kb transcript, but was still lower than in the control treatment. In Golden Promise, the large transcript was induced by long-term salt stress. The mechanism of the expression of two different size transcripts of V-ATPase B was not clear, however, the down-regulation of V-ATPase B under high salinity in Maythorpe

and up-regulation in Golden Promise could be important characters responding to salt stress between the two near isogenic cultivars.

Transcript B28 was highly homologous to the Barley (*H. vulgare* L.) *BARE*-1 (BArley RetroElement 1) retrotransposon and was isolated from short-term salt stressed Maythorpe from the differential display gel in this study. The BARE-1 retrotransposon is a major active component of the genome of barley and other *Hordeum* species with long terminal repeats (LTR) (Vicient *et al.*, 1999). Transcription of most of the active plant elements characterised to date is largely quiescent during normal development but can be induced by biotic and /or abiotic stresses (Grandbastien, 1998). As a result, LTR retrotransposons may have the ability to rapidly alter genome structure in response to environmental stress (Wessler, 2001). Unfortunately the Northern blot analysis with B28 did not show any hybridised bands, which may be because of insufficient expressed transcripts for the production of hybridisation signals.

The differential fragments B15 and B20 were homologous to a leucine zipper transcription factor. Northern blot results showed that the expression of both B15 and B20 was down-regulated by long-term salt stress in Maythorpe (Chapter 3). However, long-term salt stress induced up-regulation and over-expression of transcript B15 while the expression of B20 was not affected by long-term salt stress treatment in Golden Promise. Because B15 and B20 were complementary and B15 was longer than B20, they could play different roles in response to salt stress. In accordance with the two phase hypothesis (Munns *et al.*, 1995) plants mainly suffer ion toxicity effects under long-term salt stress. Under short-term salt stress both B20 and B15 over-expressed in Golden Promise to resist salt induced osmotic stress. Under long-term

salt stress, only B15 over-expressed in Golden Promise, the up-regulated expression of B15 could activate other structural genes against Na^+ and Cl^- accumulation in Golden Promise, whereas in Maythorpe the down-regulation of transcript B15 could mean that the expression of related structural genes might be reduced. As a result, this cultivar could be more sensitive to the accumulation of Na^+ and Cl^- ions. Therefore, the over-expression of the leucine zipper transcription factor in Golden Promise under both short-term and long-term salt stress may be an important mechanism contributing to the enhanced salt tolerance of this cultivar when compared to Maythorpe. Peng *et al.* (1999) demonstrated that the *Rht* dwarfing mutations in wheat are based on changes in a related class of gibberellin responsive transcription factor. As B15 and B20 are homologous to a leucine zipper transcription factor, their differential expression may be associated with the *GPert* mutation, which was insensitive to gibberellic acid (Pakniyat *et al.*, 1997a). A leucine zipper transcription factor was found to be inducible by light in parsley (*Petroselinum crispum*) (Feldbrügge *et al.*, 1994) and by drought and high-salinity in *A. thaliana* (Uno *et al.*, 2000). Barley cultivar Golden Promise is a *GPert* mutant produced by gamma-ray treatment. Pakniyat *et al.* (1997a) reported *GPert* as a single gene mutation located on barley chromosome 7 (5H) (Thomas *et al.*, 1984) and was relatively insensitive to exogenous gibberellic acid and salt tolerant (Pakniyat *et al.*, 1997a). A single gene mutation might have affected the expression of the leucine zipper transcription factor. The down-regulation and up-regulation of the leucine zipper transcription factor by salt in Maythorpe and Golden Promise, respectively, could be the key gene in the regulation of other functional genes such as *rbcS*, *petB*, *atpF* and a gene encoding V-ATPase B subunit in response to salinity.

Chapter 5

Effect of salt stress on photosynthesis

5.1 Introduction

Salinity is one of the important natural factors causing a severe reduction in crop production. The decline in productivity observed for many plant species subjected to excess salinity is often associated with a reduction in photosynthetic capacity (Long and Baker, 1986; Sultana *et al.*, 1999). Salt stress affects photosynthesis in two major ways; by a reduction in stomatal conductance and inhibition of rates of biochemical processes (Kalaji and Nalborczyk, 1991; Rajasekaran *et al.*, 1997; Reddy *et al.*, 1998). Kalaji and Nalborczyk (1991) reported that six barley cultivars showed different responses to salt stress with different rates of photosynthesis and transpiration. From Chapter 4, three genes related to photosynthesis, *rbcS*, *petB* and chloroplast *atpF*, were differentially expressed between Golden Promise and Maythorpe under short-term and long-term salt stress. Gene *rbcS* codes for the Rubisco small subunit, which is directly related to CO_2 assimilation (Meier *et al.*, 1995); *petB* and *atpF* code for cytochrome B6 and ATP synthase, respectively. They are associated with light reactions/electron transport (Dean *et al.*, 1989; Argüello-Astorga and Herrera-Estrella, 1998). The transcripts of these three genes were up-regulated and down-regulated by salt in Golden Promise and Maythorpe, respectively. Based on the differential expression of the above genes, the following experiments were undertaken to investigate the variations of photosynthetic rate, stomatal conductance, transpiration rate and chlorophyll concentration under salt stress to test a hypothesis that photosynthesis in Maythorpe would be less efficient than in Golden Promise in response to salinity.

5.2 Materials and Methods

5.2.1 Plant material

Seeds of Golden Promise and Maythorpe were germinated following the procedures described in section 2.2.1.1. One set of plants were grown in a growth cabinet where the growth conditions were the same as described in section 2.2.1.3 while another set of plants were grown in a glasshouse where the growth conditions were the same as described in section 2.2.1.1. The aim of having two sets of plants was to examine physiological variations in response to salinity under different growth conditions. Following germination seedlings were grown in full strength Hoagland and Arnon nutrient solution for one week, after which 150 mM NaCl was added to the nutrient solution. Plants were continuously grown in this saline medium for 21 days until the end of the expreriment. The nutrient solution was aerated and changed at weekly intervals.

5.2.2 Measurement of photosynthetic rate, stomatal conductance and transpiration rate

Measurements of photosynthetic rate, stomatal conductance and transpiration rate were performed prior to the addition of salt, while following the addition of salt, measurements were continued according to the time schedule shown in Table 5.1. All measurements were performed using a portable open gas exchange IRGA (Infra-Red Gas Analyser) system (LCA-4, ADC Ktd, Hoddesdon, UK) between 9:00 and 11:00 in the morning. The IRGA worked by passing air of a known flow rate, water vapour and CO_2 content through the leaf chamber and over the leaf. Gas exchange rates were determined from the flow rate and the difference in CO_2 concentration of air entering and leaving the chamber. Incident radiation, temperature and humidity were measured

by sensors within the leaf chamber, while a small motor within the chamber ensured that air was continually mixed. The middle part of the youngest fully expanded leaf was used for measurements with 5 replicate readings (separate plants) being taken for each treatment. When recording measurements from plants growing in the growth cabinet, to avoid changing the growth conditions, the leaf chamber was kept in the cabinet and the light of the growth cabinet was used as the light source for the leaf chamber. When measuring the plants grown in the glasshouse, the leaf chamber was used with an incident radiation unit (PLU-002, ADC Ltd), which allowed a relatively constant light intensity to be maintained. Data was stored in the data logger associated with the IRGA and downloaded to a PC for processing.

Table 5.1 Time schedule for measurement of photosynthetic rate, transpiration rate and stomatal conductance.

Measurement	Time after adding salt (hours)
1	0
2	1
3	2
4	4
5	24 (1 day)
6	48 (2 days)
7	96 (4 days)
8	144 (6 days)
9	336 (2 weeks)
10	502 (3 weeks)

All measurements were taken between 9:00 and 11:00 in the morning.

5.2.3 Chlorophyll measurement

The youngest fully expanded leaves from both control and salt stressed treatments were taken at the end of the 3 week period of exposure to salt for the determination of chlorophyll concentrations. The middle part of the youngest fully expanded leaf was weighed accurately (about 1.00 g), cut into small pieces and ground in 80 ml acetone using a pestle and mortar. After chlorophyll was extracted, the solution was carefully transferred to a 100 ml volumetric flask, the residue was washed 3 times with 5 ml acetone and each time transferred to the flask. The solution was made up to 100 ml

with acetone and mixed well. The absorbance at 645 and 663 nm was measured with a spectrophotometer and the chlorophyll concentration was calculated using the following formula:

$$\text{Chlorophyll (mg/g)} = (20.2\times A_{645} + 8.02\times A_{663})V/(L\times 1000\times W)$$

In the above formula, A = absorbance, V = volume (ml), L means light passing length of cuvette (usually taken as 1) and W = weight of sample (g).

5.3 Results

5.3.1 Variation of photosynthetic rate under salt stress

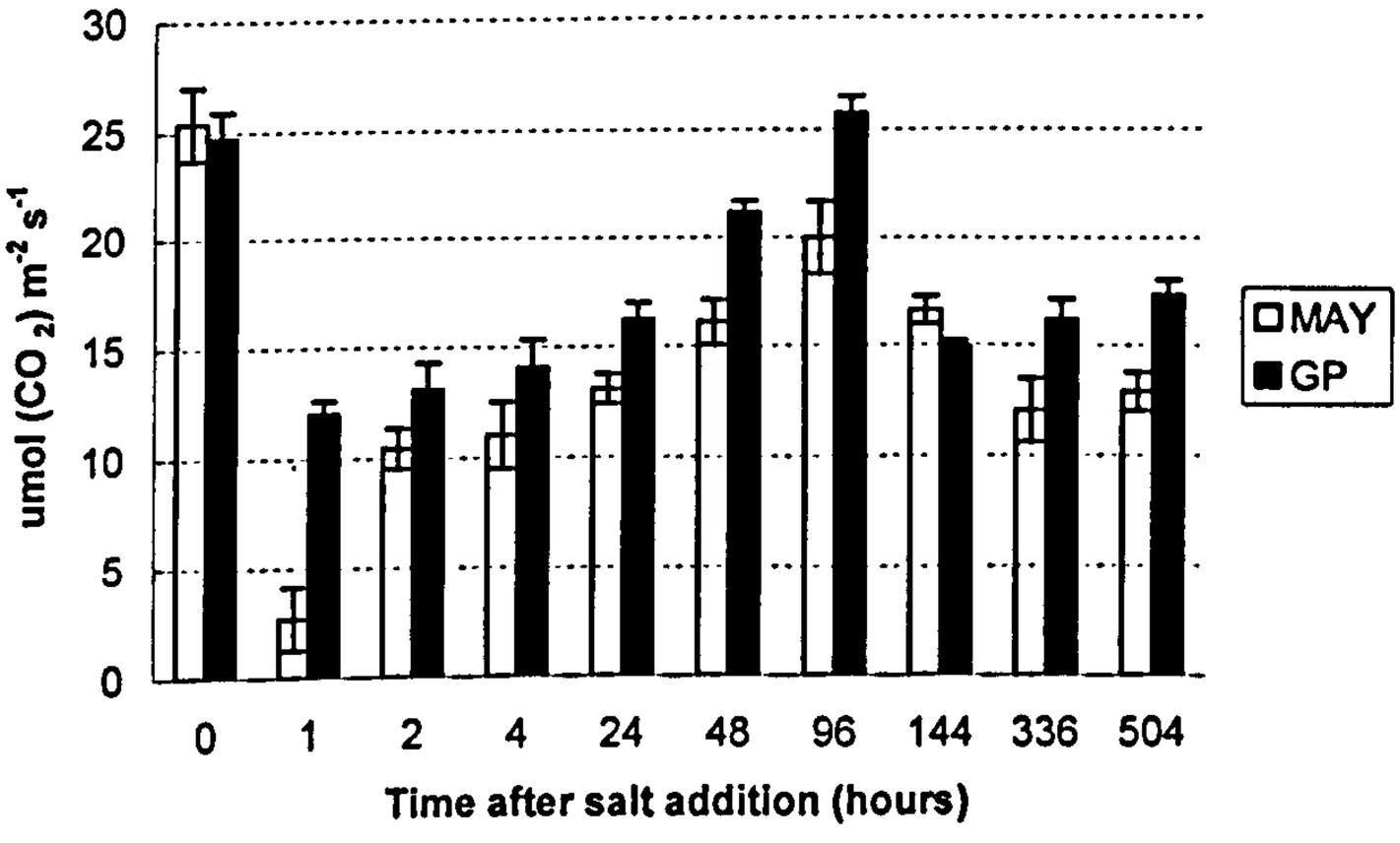
Photosynthetic rate (A) was continuously measured according to the time schedule (Table 5.1) and results from the plants grown in the growth cabinet are shown in Fig. 5.1a. Golden Promise maintained a significantly higher ($p<0.01$) photosynthetic rate than Maythorpe in the presence of salt throughout the experiment. According to the data in Fig. 5.1a, the variation of photosynthetic rates during the time course can be separated into two phases in the presence of salt. The first phase was from 1 to 96 hours following the addition of salt and Golden Promise maintained significantly higher ($p<0.01$) photosynthetic rates than Maythorpe during this period. Before the addition of salt both cultivars showed similar photosynthetic rates of about 25 μmol CO_2 m^{-2} s^{-1} . Following the exposure to salt for only 1 hour the photosynthetic rates of both cultivars were markedly reduced, but the cultivar Maythorpe showed the greater response with 89% reduction in rate compared to that at the time zero, while the corresponding reduction in Golden Promise was only 51%. In addition, at this time the photosynthetic rate of Golden Promise was about 78 % higher than that of Maythorpe. At 2 hours after the addition of salt there was a large recovery in the photosynthetic rate of Maythorpe in that it was now only about 20% lower than that of Golden

Promise. Between 2 and 96 hours after the addition of salt the photosynthetic rate of both cultivars increased gradually to such an extent that the rate of Golden Promise was at the same level as that recorded at time zero while that of Maythorpe was only 21 % lower than the time zero measurement. After that and concomitant with the start of phase 2, the photosynthetic rates of both cultivars decreased again although Golden Promise still maintained a significantly higher ($p<0.01$) photosynthetic rate than Maythorpe between 96 and 504 hours after the addition of salt. At 504 hours following the addition of salt the photosynthetic rate of Golden Promise was about 35 % higher than that of Maythorpe but only 70 % of the rate measured at time zero. Similar results were also observed for plants grown in the glasshouse (Fig. 5.1b). Golden Promise maintained a significantly higher ($p<0.01$) photosynthesis rates than Maythrope in the presence of salt throughout the experiment. The two phases in variation of photosynthetic rate were still clearly shown in Fig. 5.1b. From the beginning to 96 hours after the addition of salt, Golden Promise maintained significantly higher ($p<0.01$) photosynthetic rate than Maythrope. At the first hour following the addition of salt the photosynthetic rate of Maythorpe and Golden Promise decreased by 69 and 50 %, respectively compared to the rate at time zero. At this time Golden Promise maintained a 58 % higher photosynthetic rate than Maythorpe compared with time zero when there was no significant difference between cultivars. At 2 hours following the addition of salt the photosynthetic rate of both cultivars reached the lowest level, following which there was a gradual recovery in photosynthetic rate up to 48 hours and Golden Promise maintained a slightly higher photosynthetic rate than Maythorpe during this period. By 96 hours the photosynthetic rates of both cultivars showed a large recovery to about 65 % of the rate observed at time zero. No significant difference between cultivars was observed between 96 and 504 hours after the addition

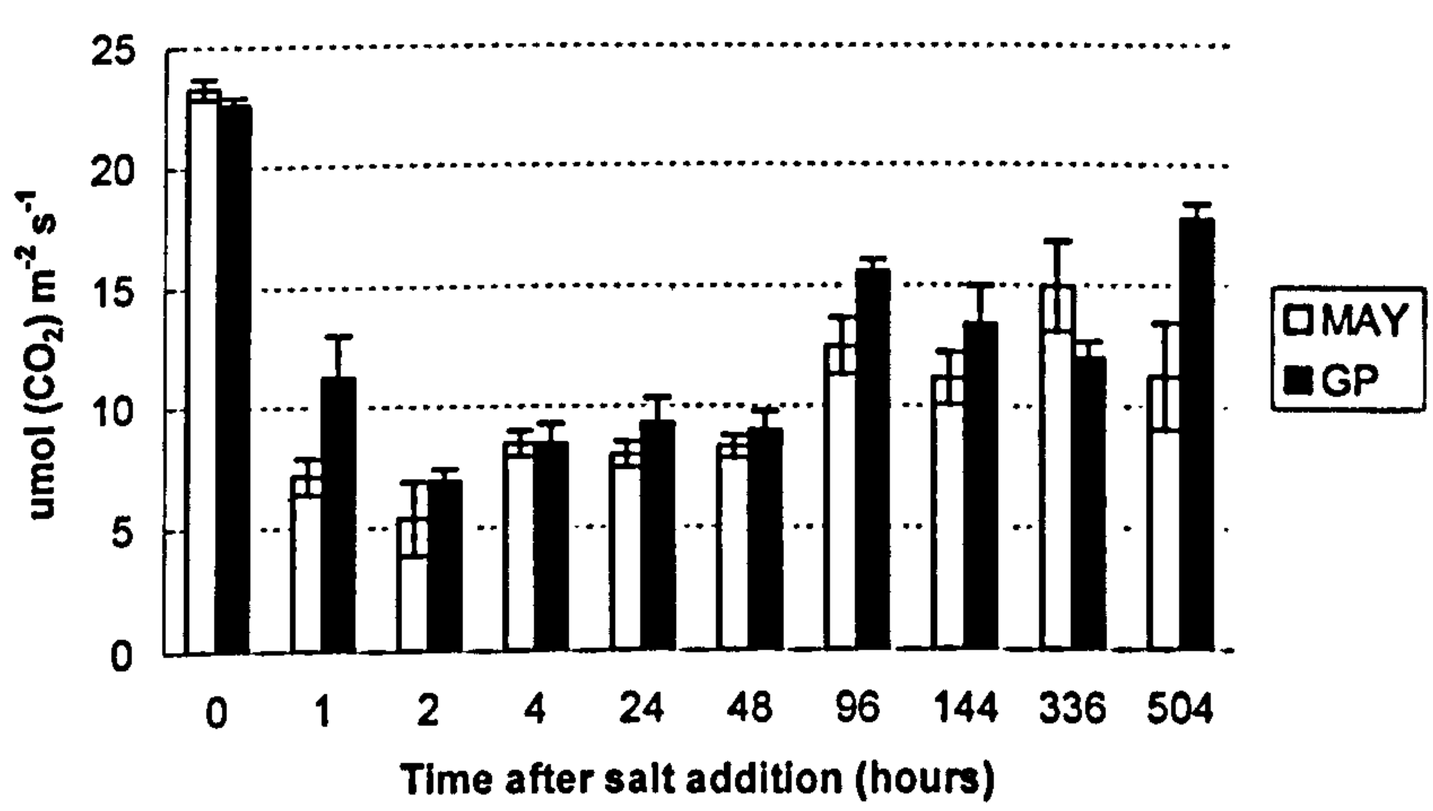
of salt. At 504 hours after the addition of salt the photosynthetic rate of Golden Promise was 36 % higher than that of Maythorpe and was 78 % of that at time zero.

Fig. 5.1 Variation in photosynthetic rate (μmol CO_2 m^{-2} s^{-1}) (means ± se) of Golden Promise (GP) and Maythorpe (MAY) following addition of 150 mM NaCl to the growth medium. For a: plants grown in growth cabinet, and b: plants grown in glasshouse.

a



b



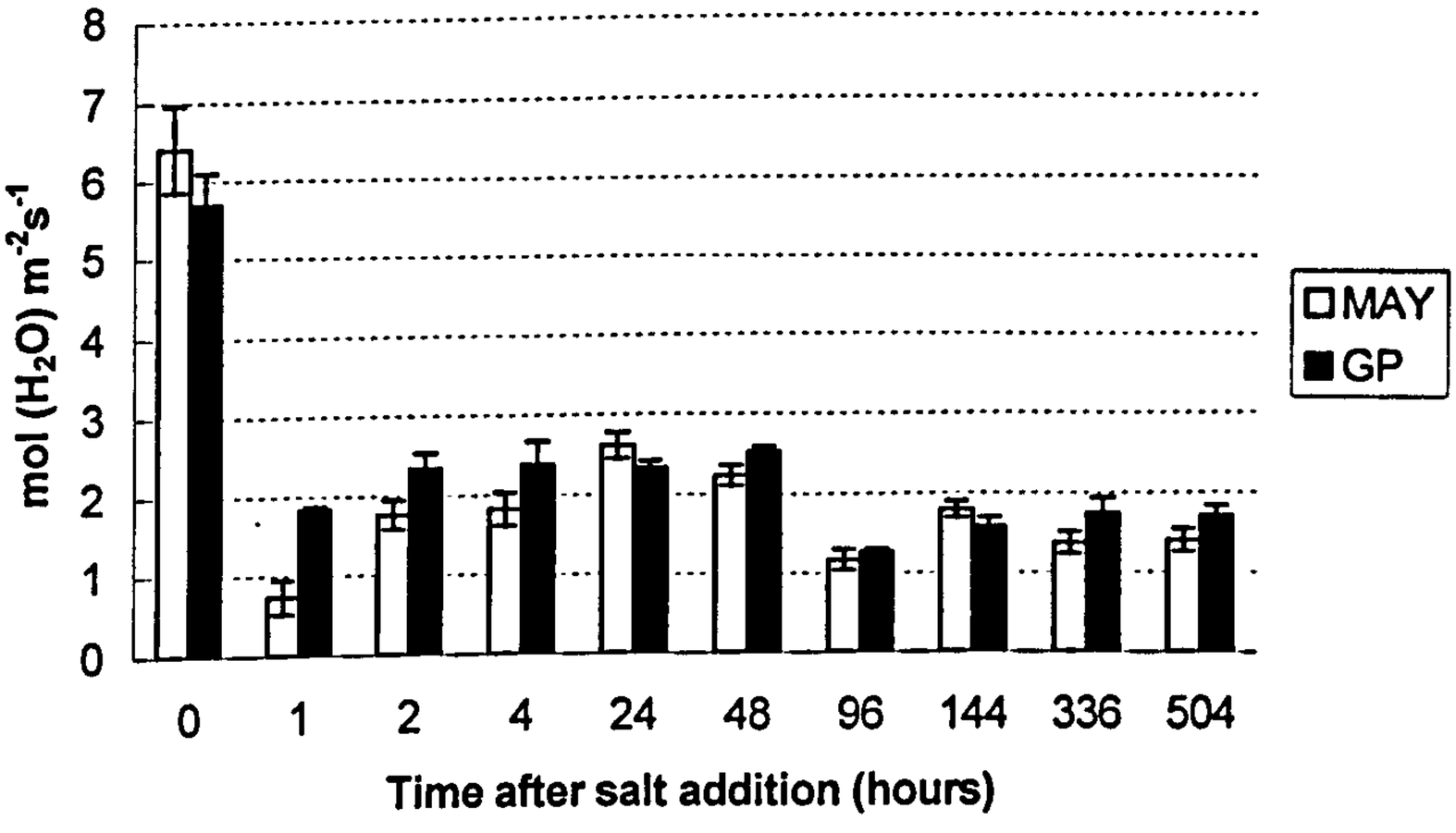
5.3.2 Variation in transpiration rate under salt stress

Salt stress resulted in a marked reduction in the transpiration rates of both Golden Promise and Maythorpe. From the plants grown in the growth cabinet (Fig. 5.2a), Maythorpe maintained a slightly higher transpiration rate than Golden Promise before the application of salt. Following the addition of 150 mM NaCl to the growth medium, like photosynthetic rate, the transpiration rates of both cultivars decreased sharply. During the first hour after the application of salt, the transpiration rate of Golden Promise was reduced from 5.70 to 1.84 mol (H_2O) m^{-2} s^{-1} (68 %) and Maythorpe from 6.42 to 0.74 mol (H_2O) m^{-2} s^{-1} (88 %), Golden Promise showed a 1.5 fold higher transpiration rate than Maythorpe. There was very little change in the transpiration rate of Golden Promise between 1 and 48 hours following the addition of salt, whereas in the cultivar Maythorpe there was a gradual increase with time till 24 hours following the addition of salt. There was a large fall in transpiration rate of both cultivars between 48 and 96 hours following the addition of salt after which time transpiration rates remained relatively stable up to end of the experiment at 504 hours following salt addition. If the time was divided into two phases as described above, Golden Promise maintained a significantly ($p<0.05$) higher transpiration rate than Maythorpe in the presence of salt from 1 to 96 hours following the addition of salt to the growth medium, but there was no significant varietal difference between the two cultivars from 96 to 504 hours following the addition of salt. The results from the plants grown in the glasshouse (Fig. 5.2b) also showed that the largest reduction in transpiration rate occurred during the first hour following the addition of salt. In this experiment transpiration rates were lower than in the growth cabinet and again Golden Promise maintained significantly ($p<0.01$) higher transpiration rates than the cultivar Maythorpe during 1 and 96 hours after the addition of salt, but there was no significant

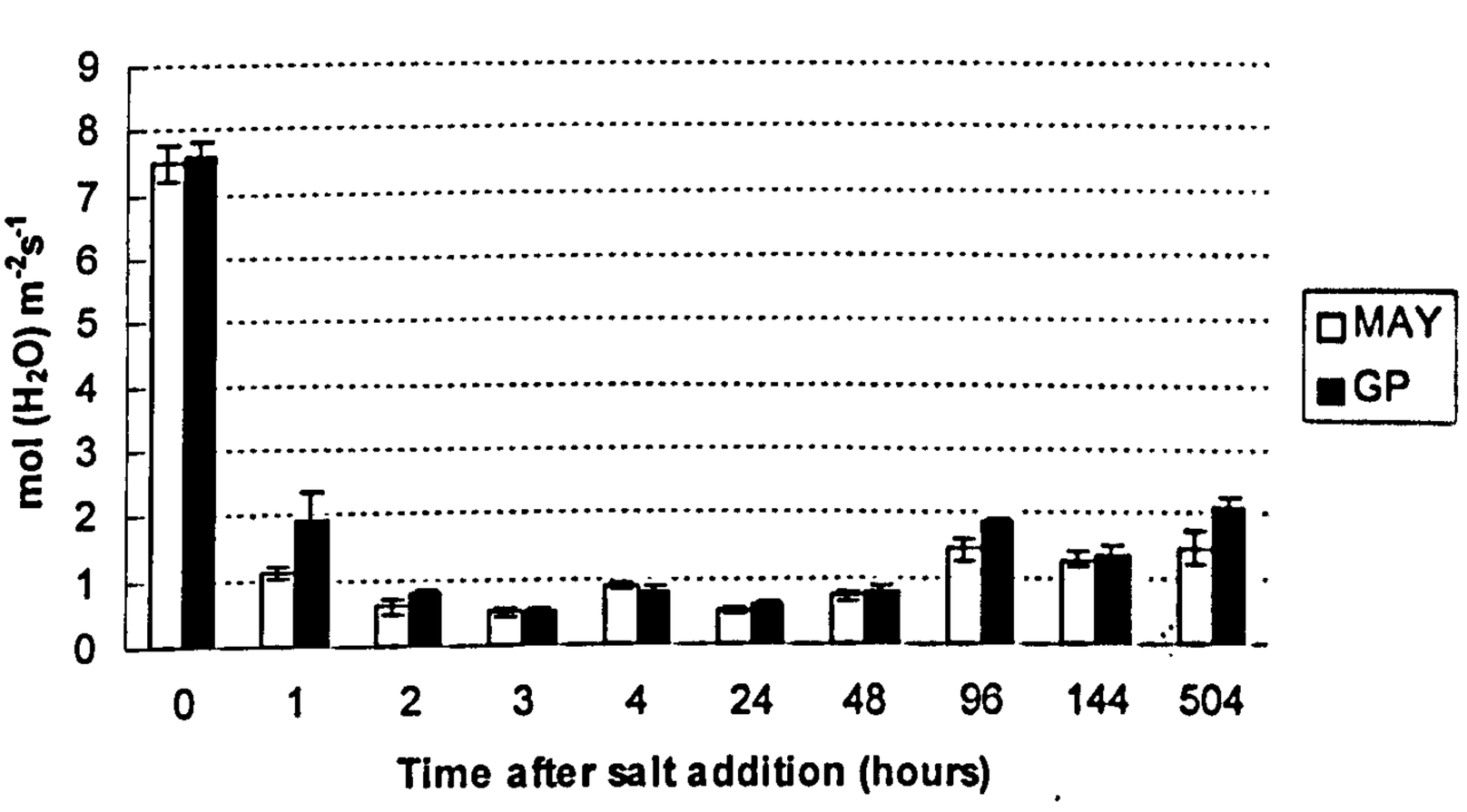
difference between cultivars during the second phase, *i.e.* 96 to 504 hours following the addition of salt.

Fig. 5.2 Variation in the transpiration rate (mol (H_2O) m^{-2} s^{-1}) (means ± se) of Golden Promise (GP) and Maythorpe (MAY) after addition of 150 mM NaCl to the growth medium. For a: plants grown in growth cabinet, and b: plants grown in glasshouse.

a



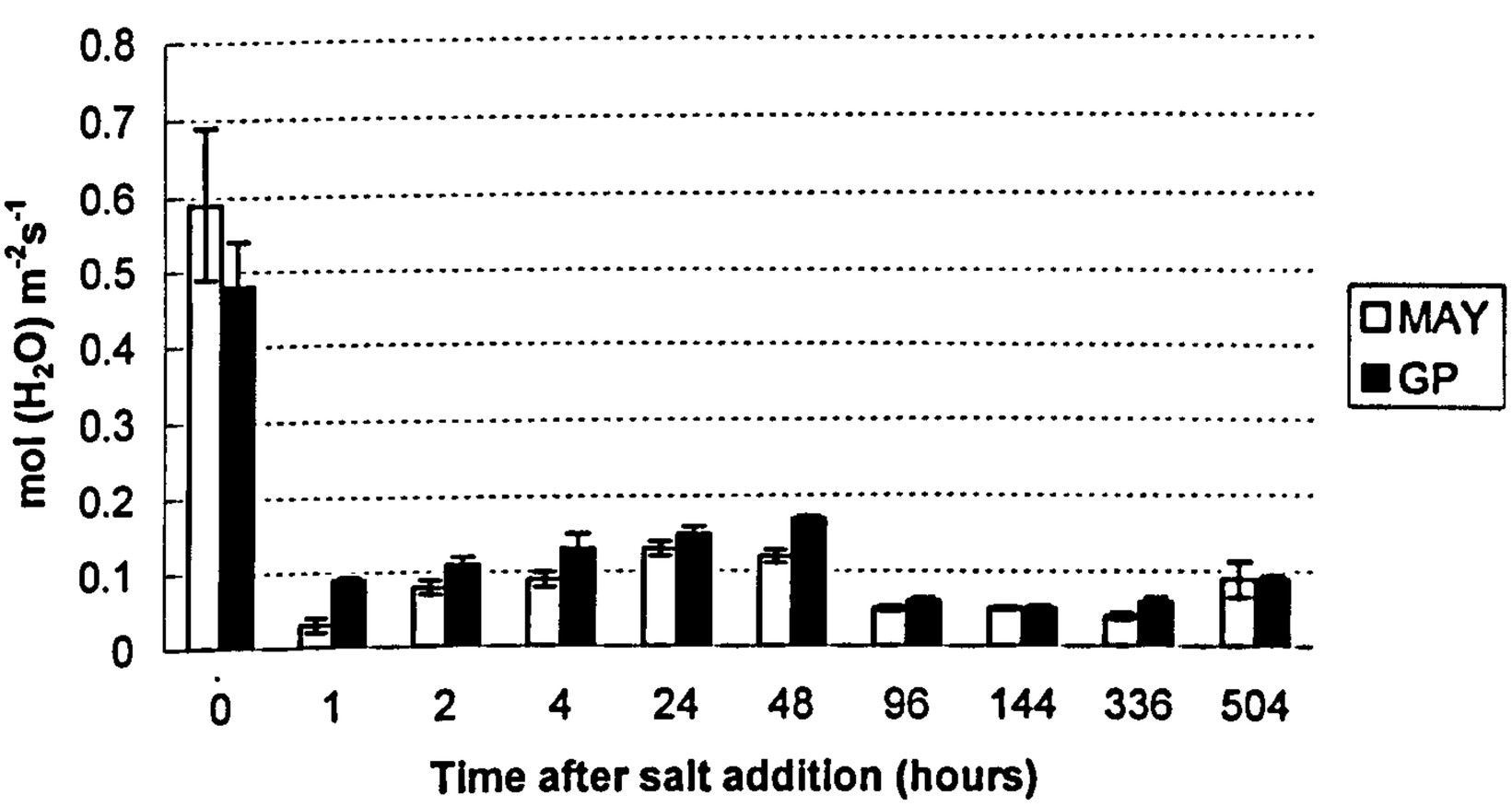
b

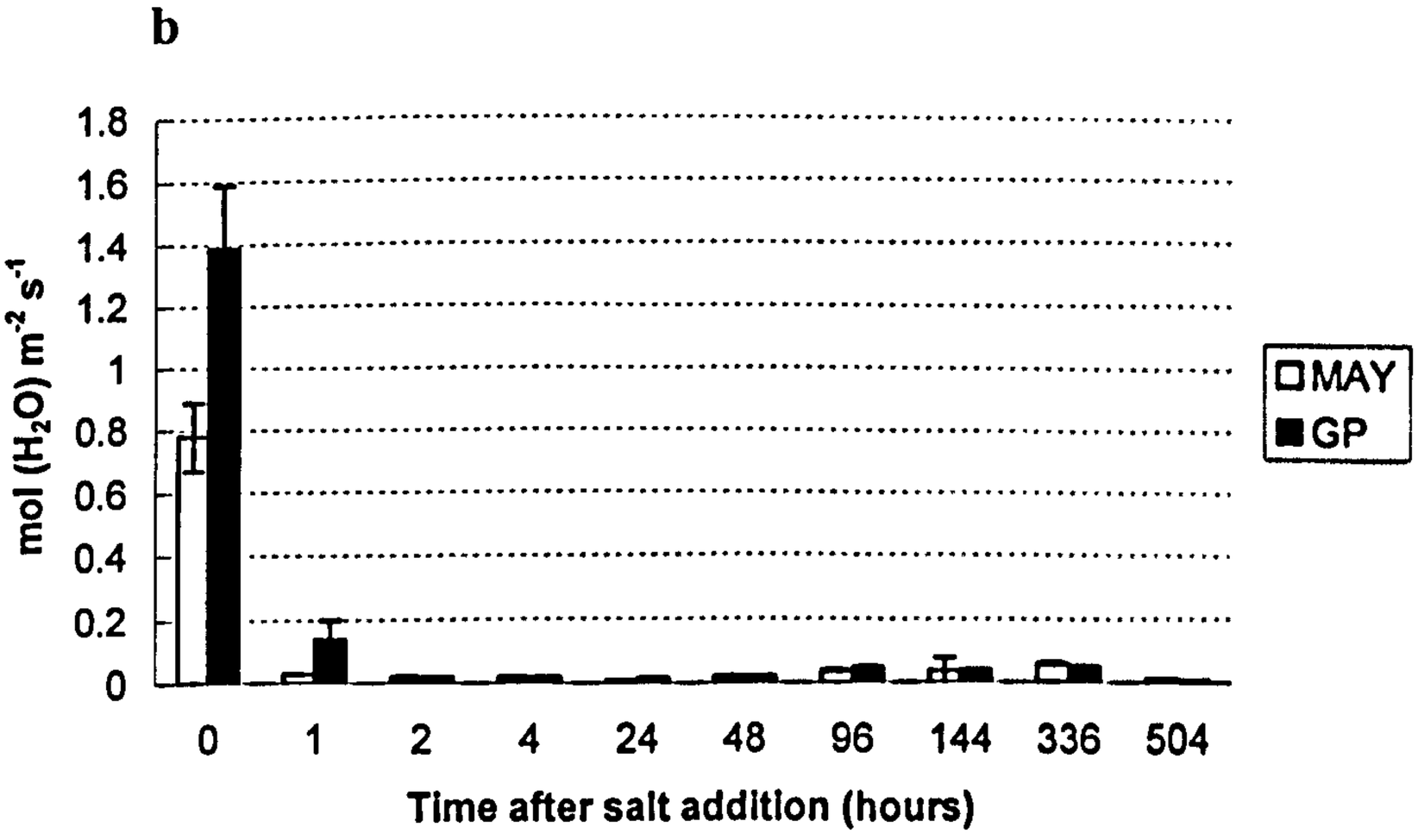


5.3.3 Variation in stomatal conductance under salt stress

The variation in stomatal conductance with time following the addition of salt to plants grown in the growth cabinet (Fig. 5.3a) showed a large reduction within 1 hour with Maythorpe being affected more than Golden Promise. In Maythorpe after 1 hour stomata were virtually closed with a conductance less than 0.5 mol (H_2O) m^{-2} s^{-1}. Between 1 and 48 hours following the addition of salt there was a gradual increase in stomatal conductance of both cultivars but even at 48 hours the stomatal conductance of Golden Promise and Maythorpe was 35 % and 20 % of that recorded at time zero, respectively. Between 48 and 96 hours after the addition of salt there was a reduction in stomatal conductance of both cultivars with very little subsequent change through to the end of the experiment. Golden Promise maintained significantly ($p<0.01$) higher stomatal conductance than Maythorpe during 1 and 96 hours following the addition of salt, but no significant difference was observed between the 96 and 504 hour period. In the greenhouse (Fig. 5.3b) prior to the addition of salt the stomatal conductance of the cultivar Golden Promise was greater than that of Maythorpe. The addition of salt severely inhibited the stomatal conductance of both cultivars such that, from 2 hours after the addition of salt through to the end of the experiment the conductance was less than 0.1 mol (H_2O) m^{-2} s^{-1}. Golden Promise again maintained a significantly ($p<0.05$) higher stomatal conductance than Maythorpe during 1 to 96 hours following the addition of salt, but there was no significant difference between cultivars from 96 hours following the addition of salt until the end of the experiment.

Fig. 5.3 Variation in stomatal conductance (mol (H_2O) m^{-2} s^{-1}) (means ± se) of Golden Promise (GP) and Maythorpe (MAY) after addition of 150 mM NaCl to the growth medium. For a: plants grown in the growth cabinet, and b: plants grown in the glasshouse.





5.3.4 Chlorophyll concentration

Golden Promise contained a higher chlorophyll concentration than Maythorpe in the youngest fully expanded leaf under both control and salt stress conditions (Table 5.2). It was also observed that salt stress caused an increase in the chlorophyll concentration of both cultivars when compared to their controls.

Table 5.2 Chlorophyll concentrations (mg/g fresh weight) (means ± se) of Golden Promise and Maythorpe with three replications after 3 weeks growth in the presence of 150 mM NaCl.

NaCl (mM)	Golden Promise mg g^{-1}fw	Maythorpe mg g^{-1}fw
0	1.74±0.10	1.47±0.03
150	2.10±0.06	1.88±0.13

5.4 Discussion

Photosynthetic capability relies on several components, *e.g.* stomatal conductance, transpiration rate, photosynthetic rate per unit leaf area, CO_2 concentration, leaf area and other environmental factors such as drought stress *etc.* Eight barley varieties were classified into tolerant, medium and sensitive to salt stress based on their variations in growth rate, photosynthetic and transpiration rate in response to increasing salinity (Kalaji and Nalborczyk, 1991). Photosynthetic rate ultimately represents the capability for CO_2 assimilation by plants. In the absence of salt there was very little difference in photosynthetic rate between the barley cultivars Golden Promise and Maythorpe. Salinity resulted in a significant reduction in photosynthetic rate in both cultivars, however, Golden Promise maintained significantly higher photosynthetic rates than Maythorpe in the presence of 150 mM external NaCl following the addition of salt. This suggests that the comparatively higher photosynthetic rate could play an important role in the enhanced salt tolerance of Golden Promise.

The variation of photosynthetic rate with salt stress can be attributed to differences in stomatal conductance (Brugnoli and Lauteri, 1991; Reddy *et al.*, 1998). Higher stomatal conductance in plants is known to aid CO_2 diffusion into leaves, which potentially could enable higher photosynthetic capacity (Seemann and Critchley, 1985), but could also result in increased water loss and dehydration. However, Ashraf (2001) reported that a reduction in stomatal conductance in response to salt stress in *Brassica* species was not significantly related to variation in photosynthetic rate. Sharma and Hall (1991) also suggested that stomatal conductance was not limiting the rate of photosynthesis in barley and sorghum, because the internal CO_2 concentrations (Ci) were not affected by increasing salinity although stomatal conductance was

severely reduced. Dunn and Neales (1993) presented similar results from *Atriplex hastate* and *H. vulgare* and suggested that stomatal closure is of secondary importance to salt tolerance. In contrast, Rawson *et al.* (1988) reported that increasing salinity resulted in a reduction in internal CO_2 concentration in barley leaf blades suggesting that stomatal conductance was limiting photosynthesis. In this study, the varietal differences in photosynthetic rate throughout the experiment might be as a result of different mechanisms during phases one and two.

According to the two-phase hypothesis (Munns, 1993; Munns *et al.*, 1995), plant growth under salt stress is initially inhibited by cellular responses to the osmotic effects of external salt, *i.e.* by responses to the decreased availability of soil water (phase 1). Golden Promise maintained significantly higher photosynthetic rate, stomatal conductance and transpiration rate than those in Maythorpe during 1 to 96 hours after the addition of salt. It is probable that stomatal effects might play an important role in the enhanced photosynthetic rate in Golden Promise when compared to Maythorpe. The significantly higher stomatal conductance in Golden Promise would enable a higher CO_2 concentration in the chloroplast resulting in a higher photosynthetic rate, and hence result in a higher transpiration rate. The ability of Golden Promise to maintain greater stomatal conductance may be as a result of greater osmotic adjustment. Wang and Ni (2000) reported that increases in glycine betaine in response to salinity occurred within three days in *Amaranthus tricol* leaves. Between 96 and 504 hours after the addition of salt Golden Promise still maintained a significantly higher photosynthetic rate than Maythorpe, but no significant varietal differences in stomatal conductance and transpiration rate were observed between the cultivars. Stomatal conductance was reduced to a very low level (less than 0.1 mol

(H_2O) m^{-2} s^{-1}) during this period. This would suggest that stomatal effects did not account for the varietal difference in photosynthesis, instead, ion toxicity and imbalance may play an important role in accordance with Munns *et al.* (1995). The fact that Golden Promise maintained significantly lower Na^+ concentrations in young leaf blades than Maythorpe (Chapter 2) with increasing salinity would result in a lower ion toxicity.

Herralde *et al.* (1998) reported that both drought and salt stress caused reductions in photosynthetic rate in *Argyranthemum coronopifolium* plants. The reduction in photosynthetic rate caused by drought could be recovered, but the reduction caused by salt could not and it was therefore suggested that ion toxicity was an important factor under salt stress. Neumann (1997) demonstrated that a high rate of salt accumulation in the mature leaves of more salt-sensitive varieties of plants such as wheat and barley would lead to toxic effects, *i.e.* accelerated leaf senescence and/or necrosis. Varietal differences during this phase should be reflected by differences in the capability to exclude and partition potentially toxic Na^+ ions. Based on investigations of variation in leaf elongation and dry matter of crops under salinity, the two-phase hypothesis indicated that varietal differences in response to salt stress should occur in phase 2 (Munns *et al.*, 1995; Neumann, 1997). However, during the first 96 hours following the addition of salt in the present study, the varietal difference was clear between Golden Promise and Maythorpe. Golden Promise maintained a significantly higher photosynthetic rate, stomatal conductance and transpiration rate than Maythorpe and the biggest difference occurred during the first hour after the addition of salt. Early varietal differences under salt stress were also reported in maize (Mladenova, 1990;

Cramer *et al.*, 1994), wheat (Kingsbury *et al.*, 1984) and *Brassica* varieties (He and Cramer, 1993).

All these early varietal differences were suggested to be caused by differential responses to the osmotic effects of NaCl, rather than differences in salt accumulation. Maythorpe is a taller plant with a bigger leaf area compared to Golden Promise. As a result, the former would be more affected by osmotic stress than the latter because more water loss would occur. As a consequence, stomatal closure would occur to restrict water loss, but at the same time may reduce CO_2 exchange resulting in a reduction in photosynthetic rate. These differences were clearly shown during the first hour after the addition of salt, when reductions in stomatal conductance, transpiration rate and photosynthetic rate were at their greatest. Recovery of stomatal conductance, transpiration and photosynthetic rate then occurred in both cultivars with time. At all times there were differences between the two cultivars, especially in photosynthetic rate. During 96 to 504 hours, however, the difference in photosynthetic rate between Golden Promise and Maythorpe became more marked, presumably as a result of differences between the two cultivars in Na^+ accumulation, especially in young actively growing tissues. Kalaji and Nalborczyk (1991) demonstrated that salt stress affects photosynthesis in two major ways: firstly limitation due to the reduced stomatal conductance, and secondly inhibition of rates of biochemical processes. Non-stomatal limitation of CO_2 assimilation capacity under salt conditions may be related to a decline in the activity of ribulose 1,5-bisphosphate carboxylase (Ziska *et al.*, 1990). Differential gene expression in young leaves under salt stress could reflect the varietal differences in photosynthesis.

In Chapter 4, the molecular analysis data showed that the down-regulation of the genes encoding the Rubisco small subunit, cytochrome B6 and ATP synthase CFO subunit I under both short-term and long-term salt stress in Maythorpe could lead to a reduction in energy supply and the capability of CO_2 assimilation. As a result, photosynthesis, *i.e.* photosynthetic rate, was markedly affected by the external salinity. In contrast, the up-regulation of the Rubisco small subunit under short-term salt stress in Golden Promise may imply that CO_2 fixation could be improved by the over-expression of this gene compared to Maythorpe. This has been shown in salt tolerant alfalfa cells under salt stress where the mRNA level of gene *rbcS* encoding Rubisco small subunit was increased (Winicow and Seemann, 1990; Winicov and Button, 1991). The up-regulation and over-expression of chloroplast gene *petB* encoding cytochrome B6 in Golden Promise under short-term and long-term salt stress, especially under long-term salt stress, could increase electron transport between PS II and PS I resulting in the production of more NADPH for photosynthesis. Winicov and Seemann (1990) reported that PS II electron transport inhibitors significantly reduced the growth of the salt-tolerant *Alfalfa* cell line HG2-N1 in the presence of salt, but not in the absence of salt. It was suggested that salt induced expression of electron transport between PS II and PS I might play a significant role in salt tolerance. The up-regulation of the *atpF* gene encoding ATP synthase under both short-term and long-term salt stress in Golden Promise would also lead to the production of more ATP for CO_2 fixation.

The difference in chlorophyll concentration between the two cultivars under salt stress could be another important feature. Golden Promise maintained higher chlorophyll concentrations than Maythorpe under control and salt stress conditions (also confirmed in Chapter 2). Salt tolerant Alfalfa cell lines contained higher chlorophyll

concentrations than salt sensitive cell lines both in the presence and in the absence of salt (Winicov and Seemann, 1990). An increased chlorophyll concentration under saline conditions is probably a concentration effect as salt stressed plants maintained smaller leaf areas than plants under control conditions.

The differences in photosynthetic rate, transpiration rate and stomatal conductance between the plants grown in the growth cabinet and the greenhouse could be related to the different growth conditions, but also to differences in conditions at the time of measurement. In the growth cabinet, conditions were consistently controlled, such as light intensity, temperature and humidity. Variations in temperature, humidity *etc* would be much greater in greenhouse than in growth cabinet both during the same day and from day to day.

It can be concluded that Golden Promise showed a greater ability to maintain higher photosynthetic rate, stomatal conductance and transpiration rate during 1 to 96 hours and a higher photosynthetic rate during 96 to 504 hours after the addition of salt compared to Maythorpe. These physiological varietal differences supported the differential expression of the three photosynthetic genes *rbcS, petB and atpF* encoding the Rubisco small subunit, cytochrome B6 and chloroplast ATP synthase CFO subunit I, respectively, between Golden Promise and Maythorpe.

Chapter 6

General discussion and future work

6.1 General discussion

Barley is one of the most salt tolerant glycophytic plant species (Ayers *et al.*, 1952; Bole and Wells, 1979). Numerous variations in salt tolerance among barley cultivars have been reported in the literature (Huang and Van Stevenick, 1989; Kalaji and Nalborczyk, 1991). The barley cultivar Golden Promise is a direct mutant (*GPert*) of Maythorpe, which was produced by gamma-ray irradiation (Sigurbjornsson and Micke, 1969). These two cultivars with isogenic backgrounds exhibit different growth characteristics and different responses to salinity (Forster *et al.*, 1994; Pakniyat *et al.*, 1997a; Forster, 2001). The *GPert* mutation, therefore has pleiotropic effects on growth and salt tolerance. With the exception of differences in Na^+ concentration in the shoot tissue (Pakniyat *et al.*, 1997b) little was known of the physiological or molecular basis of the *GPert* trait.

In the present study, increasing salinity caused a significant reduction in the growth of both Golden Promise and Maythorpe, with Maythorpe being affected to a greater extent than Golden Promise. Generally, plant growth is largely dependent on the capacity to assimilate CO_2 into carbohydrates *via* photosynthesis. To maintain a high capacity for photosynthesis under saline conditions the presence of young actively growing tissues and the maintenance of chlorophyll concentration are important components. Golden Promise maintained higher YLB:OLB ratios and chlorophyll concentrations than Maythorpe with increasing external NaCl concentrations in the

growth medium. Therefore, Golden Promise possessed advantages for maintaining photosynthesis and growth when compared to Maythorpe under saline conditions.

Concerning the effects of individual salt treatments on growth, both cultivars were found to grow relatively well under low salt concentrations, *e.g.* 50 mM external NaCl, while 200 mM NaCl was observed to be too severe for both cultivars. Therefore 150 mM NaCl was an optimum concentration for determining varietal differences. This concentration is similar to the 175 mM NaCl, which was used by Forster *et al.* (1994) and Pakniyat *et al.* (1997a) in previous studies.

Measurements taken from the first fully expanded leaves exposed to 150 mM external NaCl showed that Golden Promise maintained a significantly higher photosynthetic rate than Maythorpe throughout the duration of the salt treatment, *i.e.* 21 days. Golden Promise also maintained significantly higher chlorophyll concentrations in the young leaves and significantly higher stomatal conductance during the period from 1 to 96 hours after the addition of salt than Maythorpe. There is good evidence that these physiological varietal differences in photosynthesis under salt stress between the cultivars result from variation in expression of paticular genes. Three photosynthetic gene transcripts encoding the Rubisco small subunit, cytochrome B6 and ATP synthase CFO subunit I were differentially expressed between Golden Promise and Maythorpe under salt stress (150 mM NaCl). All these gene transcripts were up-regulated in Golden Promise and down-regulated in Maythorpe by salinity. The Rubisco small subunit is involved in CO_2 assimilation, whilst cytochrome B6 and ATP synthase CFO subunit I are involved in electron transport leading to the production of energy in the form of NADPH and ATP in the chloroplast. The up-regulation of the

Rubisco small subunit and cytochrome B6 and ATP synthase CFO subunit I by salt could result in reducing the effects of salt on the photosynthesis system in Golden Promise. In contrast, the down-regulation of the three gene transcripts in Maythorpe implied that the photosynthetic system would be more severely damaged by salt compared to that of Golden Promise. These molecular differences in gene expression are in agreement with the physiological data obtained where Golden Promise maintained a significantly lower Na^+ in young tissues and a significantly higher photosynthetic rate and chlorophyll concentration than Maythorpe under salt stress. Maintenance of a high photosynthetic rate under salt stress could be an important mechanism in the enhanced salt tolerance in Golden Promise compared to Maythorpe.

Ion homeostasis plays an important role in salt tolerance in plants (Serrano *et al.*, 1999; Hasegawa *et al.*, 2000). When the NaCl concentration increases in the surrounding environment it is crucial for a plant to maintain a low Na^+ concentration in the cytoplasm because cytosolic Na^+ concentrations in excess of about 100 mM would be cytotoxic (Amtmann & Sanders, 1999). To maintain low cytosolic Na^+ in plants, V-ATPase and vacuolar pyrophosphatase play an important role in energisation of endomembranes by pumping H^+ into the vacuoles. This proton motive force is important in maintaining secondary active transport (Davies, 1997b, Dietz *et al.*, 2001), such as vacuolar Na^+/H^+ antiporters. Vacuolar Na^+/H^+ antiporters exchange Na^+ for protons across a tonoplast membrane resulting in extrusion of excess Na^+ from the cytoplasmic compartment into vacuoles (Krulwich, 1983; Grinstein *et al.*, 1989; DuPont, 1992; Forgac, 1998). The over-expression of a vacuolar Na^+/H^+ antiporter improved salt tolerance in *Arabidopsis* (Apse *et al.*, 1999). Since vacuolar pyrophosphatase is an alternative or additional energisation of the tonoplast in case of

insufficient V-ATPase activity, maintaining the activity of the V-ATPase under environmental stress is crucially important for plants to survive (Dietz *et al., 2001). In* barley roots the V-ATPase may be involved in the sequestration of Na^+ ions in the vacuole (Garbarino and DuPont, 1989; DuPont *et al.*, 1990). From this study, a gene encoding the V-ATPase B subunit was up-regulated in Golden Promise and down-regulated in Maythorpe by both short-term and long-term salt stress. These results suggested that the over-expression of the V-ATPase B subunit induced by salt in Golden Promise could potentially generate a high electrochemical potential, which can be used to drive vacuolar Na^+/H^+ antiporters to sequester excess Na^+ into the vacuoles thereby reducing Na^+ toxicity. The significantly higher photosynthetic rate in Golden Promise than in Maythorpe under salt stress could be related to the up-regulation of the V-ATPase B subunit. On the contrary, in Maythorpe, the reduction in the expression of the V-ATPase B subunit could lead to Na^+ accumulation in the cytoplasm. A raised Na^+ concentration might disturb metabolism in the cell and disrupt the membrane proton gradient. From the physiological data, Maythorpe contained significantly higher Na^+ concentrations in the young actively growing tissues than Golden Promise with increasing salinity. The higher Na^+ concentration resulted in a disturbed ion balance so that K^+/Na^+ and Ca^{2+}/Na^+ ratios were significantly lower in Maythorpe than in Golden Promise.

Munns (1993) hypothesised that plant growth is initially inhibited (phase 1) by cellular responses to the osmotic effects of external salt. In a later, second response (phase 2), growth is further inhibited by the toxic effects of excessive salt accumulation within the plant. Based on this two-phase model, varietal differences between plant species in salt tolerance was suggested to only occur during phase 2 (Munns *et al.*, 1995;

Neumann, 1997). However, comparing the two near-isogenic barley cultivars Golden Promise and Maythorpe in the present study, early differences in photosynthetic rate, transpiration rate and stomatal conductance were clearly visible, even within one hour after the addition of salt to the growth medium. The salt tolerant cultivar Golden Promise maintained a significantly higher photosynthetic rate, transpiration rate and stomatal conductance than Maythorpe during the early stages following exposure to salt. He and Cramer (1993) detected early varietal differences in leaf growth response to salinity in two *Brassica* varieties after 3 days but Na^+ and Cl^- concentrations in leaves were not different. Cramer *et al.* (1994) also reported early varietal differences in leaf elongation rates of maize within 5 hours and 9 days of salinisation. The variety which was more sensitive to early growth inhibition by salinity accumulated significantly less Na^+ than the salt-resistant variety. Thus the early response of these species to salinity was not proportionally related to salt accumulation. If Na^+ accumulation is not to account for the early varietal difference, osmotic stress induced by salinity is the most possible cause. Among osmoregulatory compounds, glycine betaine is one of the important compatible solutes in barley in adaptation to osmotic stress (Grumet and Hanson, 1986; Kishitani *et al.*, 1994). It was reported that glycine betaine acts as an osmoprotectant by stabilising the quaternary structure of proteins against the adverse effects of high salinity and extreme temperature (Gorham, 1995). In photosynthetic systems, for example, betaine efficiently protects various components of the photosynthetic machinery, such as the Rubisco and the PS II complex (Papageorgiou and Murata, 1995). When both cultivars were exposed to 150 mM external NaCl, Golden Promise maintained significantly higher stomatal conductance and transpiration rates than Maythorpe, and presumably higher cell turgor. It could be hypothesised that Golden Promise possessed a greater ability to

quickly produce compatible solutes such as glycine betaine in response to salt stress compared to Maythorpe, which slowed down water loss from cells. Thus Golden Promise maintained higher stomatal conductance and transpiration rates, which would enable greater CO_2 exchange and as a result, this cultivar was able to maintain higher photosynthetic rates than Maythorpe during this period. Tsiantis and co-workers (1996) reported that transcriptional activation of the V-ATPase subunit c was shown in leaves and roots of *Mesembryanthemum crystallinum* treated with 350 mM NaCl for 24 hrs. An increase of the V-ATPase subunit c mRNA levels was detected in fully expanded leaves of *M. crystallinum* after only 8 hrs treatment with 400 mM NaCl (Löw *et al.*, 1996). In the present study, the V-ATPase B subunit was up-regulated and down-regulated after 24 hrs treatment by 150 mM NaCl in Golden Promise and Maythorpe, respectively (Wei *et al.*, 2002b). Therefore the early varietal differences in response to salinity between Golden Promise and Maythorpe could be mediated by induced differential gene expression. In phase 2 (96-504 hours following the addition of salt) Golden Promise maintained significantly higher photosynthetic rates than Maythorpe, but no significant difference in stomatal conductance was observed between the cultivars during the later phase. This could mean that stomatal conductance was not a major factor accounting for the differences in photosynthetic rates between the two cultivars during phase 2. While Golden Promise contained significantly lower Na^+ concentrations in young leaves than Maythorpe, the difference in Na^+ accumulation could be an important factor in relation to varietal differences in photosynthetic rates in phase 2.

A barley leucine zipper transcription factor was differentially expressed between Golden Promise and Maythorpe in relation to salt stress. This leucine zipper could be a

key gene contributing to the difference in salt tolerance between the two cultivars, as this class of protein can regulate the expression of other genes in response to salt. Initially, this leucine zipper gene was over-expressed in Maythorpe and under-expressed in Golden Promise under control conditions. The over-expression (up-regulation) of the barley leucine zipper transcription factor in Golden Promise and down-regulation in Maythorpe under short-term and long-term salt stress indicated that this transcription factor could play an important role in the different responses to salt stress between the cultivars. Related leucine zipper transcription factors are thought to activate other inducible genes under salt stress (McKnight, 1991; Uno *et al.*, 2000). Therefore there is a probability that the differential expression of the barley leucine zipper could regulate other salt inducible genes to respond to salinity stress, such as the genes for the Rubisco small subunit, cytochrome B6, ATP synthase CFO subunit I and the V-ATPase B subunit. Peng *et al.* (1999) reported that *Rht* (reduced height) height-regulating genes encoding proteins that resemble nuclear transcription factors containing leucine heptad repeats in wheat, reduce plant height and responses to gibberellin. The *GPert* mutant Golden Promise also showed a reduced response to gibberellic acid (Pakniyat *et al.*, 1997b). Possible pleiotropic effects on growth and salt tolerance might therefore be mediated by leucine zipper transcription factors. This hypothesis would be consistent with a mutation that maps to a single gene locus producing several physiological and morphological changes in barley. However, it is not yet clear at which point in the regulatory cascade of salt responses B15/B20 operates. Conceivably, alterations in expression of this gene may be the direct or indirect response to changes in the activities of other genes, such as the V-ATPase, that influence cation concentrations.

In summary, the two isogenic barley cultivars Golden Promise and Maythorpe showed differences in both growth habit and salt tolerance with Golden Promise being comparatively more salt tolerant than its parent Maythorpe. As Golden Promise is a direct mutant from Maythorpe by gamma-ray irradiation, its salt tolerance trait should be a result of the *GPert* mutation. Although a number of genes are likely to be involved in salt tolerance in plants, a smaller number of transcription factors may be much more important in the regulation of the expression of many structural genes in response to salinity. Under salt stress, the expression of the leucine zipper (B15/B20) gene showed a different pattern between the two cultivar. It was up-regulated in Golden Promise and down-regulated in Maythorpe by both short-term and long-term salt stress. The difference in the expression of the leucine zipper transcription factor in response to salinity may regulate the expression of other genes, such as genes encoding the Rubisco small subunit, cytochrome B6, chloroplast ATP synthase, and the V-ATPase B subunit. Dietz and his coworkers (2001) suggested that under stress conditions such as salinity, drought, cold, acid stress, anoxia, and excess heavy metals in the soil, survival of cells depends strongly on maintaining or adjusting the activity of the V-ATPase. The V-ATPase B subunit was up-regulated in Golden Promise and down-regulated in Maythorpe under both short-term and long-term salt stress, which suggested that Golden Promise could be more efficient in excluding excess Na^+ from the cytoplasmic compartments into the vacuoles than Maythorpe. As a result, Golden Promise maintained significantly lower Na^+ concentrations in the young actively growing tissues than Maythorpe. Furthermore, Golden Promise also maintained significantly higher K^+/Na^+ and Ca^{2+}/ Na^+ ratios than Maythorpe. The genes encoding the Rubisco small subunit, cytochrome B6 and ATP synthase were down-regulated in Maythorpe by both short and long-term salt stress. However, in Golden Promise, the

Rubisco small subunit was up-regulated by short-term salt stress and cytochrome B6 was up-regulated by both short and long-term salt stress. Because these are photosynthesis related genes, the differential expression between the two cultivars was closely related to the difference in photosynthesis, Golden Promise maintaining significantly higher photosynthetic rates than Maythorpe at 150 mM external NaCl. Therefore, Golden Promise is comparatively salt tolerant compared to the isogenic parent Maythorpe, and Golden Promise maintained a higher comparative growth than Maythorpe with increasing salinity.

6.2 Future work

1) Full cDNAs of each differential transcript could be isolated by using RACE (rapid amplification of cDNA ends) methodology (Frohman *et al.*, 1988). The functions of the differential transcripts, particularly for those without significant homologues from GenBank could be identified with the sequences.

2) Transformation of the leucine zipper transcription factor (B15/B20) to understand the regulation of this gene in both Golden Promise and Maythorpe in relation to salt stress. This analysis would show the relationships between the leucine zipper transcription factor and the Rubisco small subunit, cytochrome B6, ATP synthase and V-ATPase genes.

3) Salt stress was dominated by osmotic stress during phase 1, Golden Promise maintained significantly higher photosynthetic rates and

transpiration rates than Maythorpe during phase 1 and several genes were up-regulated in Golden Promise under short-term salt stress. Investigation of the differences in osmo-regulatant concentrations such as glycine betaine and proline in tissues of both cultivars under salt stress could supply important evidence in understanding the enhanced salt tolerance in Golden Promise.

4) The correlation of cloned DNA sequences with classically defined loci for salt tolerance in barley using the mapping populations available at Scottish Crop Research Institute.

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APPENDIX

1. 10 × TBE buffer:

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml
dH_2O	make up to 1000 ml

2. Polyacrylamide gel loading buffer (10 ml):

Deionised formamide	9.8 ml
0.5 M EDTA (pH 8.0)	0.2 ml
Xylene cyanol FF	0.0025 g
Bromophenol blue	0.0025 g

3. DNA loading buffer (10 ml):

Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Sucrose	4 g
dH_2O	make up to 10 ml

4. *IPTG stock solution (0.1 M): 1.2 g IPTG* dissolved in 50 ml of dH_2O, filter-sterilise and stored at 4°C.

5. X-Gal stock solution (2 ml): 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside dissolved in 2 ml of N,N'-dimethylformamide. The container was covered with aluminium foil and stored at -20 °C.

6. LB medium (per liter):

Bacto®-tryptone	10 g
Bacto®-yeast extract	5 g
NaCl	5 g

To 950 ml of deionised water, the above chemicals were added and shaked until the solutes dissolved. The pH was adjusted to 7.0 with 5M NaOH. The volume of the solution was adjusted to 1 l with dH_2O and sterilised by autoclaving for 20 min.

7. LB plates with ampicillin: 15 g agar was added to 1 l of LB medium and autoclaved. The medium cooled to 50 °C before adding ampicillin to a final concentration of 100 µg/ml. 30-35 ml of medium was poured into 85 mm petri dishes. The plates were stored at 4 °C for up to 1 month.

8. LB plates with ampicillin/IPTG/X-Gal: 15 g agar were added to 1 l of LB medium and autoclaved. The medium was allowed to cool to 50 °C before adding ampicillin, IPTG and X-Gal to final concentrations of 100 µg/ml, 0.5 mM and 80 µg/ml, respectively. 30-35 ml of medium was poured into 85 mm petri dishes. The plates were stored at 4 °C for up to 1 month.

9. SOC medium (100 ml):

Bacto®-tryptone	2.0 g
Bacto®-yeast extract	0.5 g
1 M NaCl	1 ml
1 M KCl	0.25 ml
2 M Mg^{2+} stock	1 ml
2 M glucose (filter-sterilised)	1 ml

Bacto-tryptone, Bacto-yeast extract, NaCl and KCl were added to 97 ml distilled water. This was stirred to dissolve and pH was adjusted to 7.0, autoclaved and cooled to room temperature. Before using, 2 M Mg^{2+} stock and 2 M glucose were added and brought to 100 ml with sterilised distilled water. The complete medium was filtered through a 0.2 µm filter.
2 M Mg^{2+} stock solution: 20.33 g $MgCl_2 \cdot 6H_2O$ and 24.65 g $MgSO_4 \cdot 7H_2O$ dissolved and make up to 100 ml with dH_2O (filter-sterilised).

10. 20×SSC: 175.3 g of NaCl and 88.2 g of sodium citrate were dissolved in 800 ml H_2O. The pH was adjusted to 7.0 with 10 M NaOH. The volume was adjusted to 1 l with dH_2O and sterilzed by autoclaving.

11. 10 % sodium dodecyl sulfate (SDS): 100 g of electrophoresis-grade SDS was dissolved in 900 ml of H_2O and heated to 68 °C to assist dissolution. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl and the volume was adjusted to 1 l with H_2O.

12. 50×Denhardt's reagent:

Ficoll	1.2 g
Polyvinylpyrrolidone	1.2 g
Bovine serum albumin	1.2 g
dH_2O	Made up to 120 ml
Sterilised by filtering	

13. Formaldehyde gel-loading buffer:

0.5 M EDTA (pH 8.0)	20 µl
Gycerol	5 ml
Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
DEPC-treated H_2O	Made up to 10 ml

14. 20×SSPE: 175.3 g of NaCl, 27.6 g of $NaH_2PO_4 \cdot H_2O$ and 7.4 g of EDTA were dissolved in 800 ml of H_2O. The pH was adjusted to 7.4 with NaOH and the volume was adjusted to 1 l with H_2O and sterilised by autoclaving.

15. Concentrations of RNA of barley tissues measured by spectrometer. The formula for calculation is: µg RNA/ml = A_{260} × Dilution factor × 40. Dilution factor = 200.

Cultivar	Tissue	Treatment	A_{260}	µg RNA/ml	A_{260}/A_{280}
Golden Promise	leaves	CK	0.083	664	2.18
	leaves	150-1d	0.064	512	2.91
	leaves	150-4w	0.071	568	2.70
	leaves	Rec	0.065	520	2.95
	leaves	B toxicity	0.070	560	3.18
	leaves	Mannitol	0.041	328	5.13
Maythorpe	leaves	CK	0.061	488	2.03
	leaves	150-1d	0.047	376	4.70
	leaves	150-4w	0.045	360	2.65
	leaves	Rec	0.088	704	2.67
	leaves	B toxicity	0.079	632	2.93
	leaves	Mannitol	0.034	272	8.50
Golden Promise	root	CK	0.037	296	4.63
	root	150-1d	0.037	296	5.29
	root	150-4w	0.142	1136	2.03
	root	Rec	0.063	504	3.15
	root	B toxicity	0.042	336	4.20
Maythorpe	root	CK	0.060	480	3.0
	root	150-1d	0.055	440	2.90
	root	150-4w	0.032	256	2.78
	root	Rec	0.055	440	3.24
	root	B toxicity	0.066	528	2.24

16. TBE buffer (5 × stock solution): 54 g Tris base, 27.5 g boric acid were dissolved in 900 ml dH_2O and 20 ml 0.5 M EDTA (pH 8.0) was added and the volume was adjusted to 1000 ml.

17. STET buffer: 80 g sucruose and 50 g Triton X-100 were dissolved in 700 ml dH_2O, 100 ml of 0.5 M Tris base (pH 8.0) and 100 ml of 0.5 M EDTA (pH 8.0) were added and the pH was adjusted to 8.0 and sterilised. The solution was stored at 4 °C.

18. Lysozyme/RNase mixture: 100 mg lysozyme was dissolved in 8 ml H_2O, 10 mg RNase and 1 ml of 0.5 M Tris-HCl (pH 8.0) were added and the volume was adjusted to 10 ml with H_2O. The solution was stored at -20 °C in small aliquots.

19. 10 × RQ1 RNase-free DNase reaction buffer: 400 mM Tris-HCl (pH 8.0), 100 mM $MgSO_4$ and 10 mM $CaCl_2$.

20 RQ1 RNase-free DNase stop solution: 20 mM EGTA (pH 8.0).

20. MMLV reverse transcriptase 5 × reaction buffer: 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$ and 50 mM Dithiothreitol (DTT).

21. Cell resuspension solution: 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A.

23. Cell lysis solution: 0.2 M NaOH, 1 % SDS.

24. Neutralization solution: 1.32 M potassium acetate (pH 4.8).

25. Column wash solution: 80 mM potassium acetate, 8.3 mM Tris-HCl (pH 7.5), 40 µM EDTA.